PHD

Formulation Optimization for the Topical Delivery of Active Agents in Traditional Medicines

Thitilertdecha, Premrutai

Award date:
2013

Awarding institution:
University of Bath

FORMULATION OPTIMIZATION FOR THE TOPICAL DELIVERY OF ACTIVE AGENTS IN TRADITIONAL MEDICINES

PREMRUTAI THITILERTDECHA

A thesis submitted for the degree of Doctor of Philosophy

University of Bath

Department of Pharmacy and Pharmacology

April 2013

Table of Contents

Acknowledgements

First and foremost, I would like to thank my supervisors, Prof. Richard H Guy and Dr. Michael G Rowan, for their valuable guidance, excellent supervision, and endless support throughout my studies. Without them, I would not have this priceless experience in Bath and this work would have not been possible.

I also gratefully acknowledge Faculty of Medicine Siriraj Hospital, Mahidol University for the financial support.

I am really thankful to Assoc. Prof. Tawee Laohapand and Assoc. Prof. Pravit Akarasereenont for giving me the opportunity to study the doctoral degree and their great support.

I also express my appreciation for kindly advice, important research and analytical techniques from Sarah Cordery. The help and guidance from Dr. Begoña Delgado-Charro was greatly appreciated. I thank Dr. Timothy J Woodman for his help in NMR spectrometry, Pascal Loizeau for his IT help, and Don Perry, Ewan Basterfield, Kelvin Smith, Jo Carter, Susan Alston and Jantanee Wattanarangsan for their technical support.

I especially thank Dr. Luis Nogueiras Nieto for his stimulating academic discussion, great moral support and precious friendship, and Dr. Natalie Belsey for her fantastic help in academic and language comments, and friendliness.

My life in Bath has been amazing thanks to all my wonderful friends, Kitiya Tangpoonsinthana, Chiau Ming Long, Dr. Liz O'Donovan, Luis Rodrigo Contreras, Wing Sin Chiu, Valeria Pergament, Dr. Amit Nathubhai, Assoc. Prof. Guilherme Gelfuso, Dr. Quan Yang, Irena-Jagoda Tomaszewska, Christopher A Brimson, Dr. James M Brimson and Jade Lermcharaskul, and all my colleagues, past and present.

Finally, I forever thank my parents and my siblings for their love, affection, infinite patience, encouragement and tremendous support at all times.

Abstract

In Thailand, *Acanthus ebracteatus* Vahl and *Clerodendrum petasites* S. Moore have been prescribed to treat skin diseases, such as rash, abscess, and urticaria, for at least 30 years. However, there is limited scientific support and no clinical trials that identify and verify the compounds that elicit useful pharmacological effects following their topical delivery.

Vanillic acid was identified for the first time in *A. ebracteatus* together with verbascoside; furthermore, nine phenolic compounds, vanillic acid, 4-coumaric acid, ferulic acid, verbascoside, nepetin, luteolin, chrysin, naringenin, and hesperetin, and two reported, apigenin and hispidulin, were found in *C. petasites*. *C. petasites* (CP) was therefore chosen as the principal plant to be studied in this thesis. Hispidulin was quantified as a predominant compound, being present at 39 µmol/g (1.2% w/w) in a dried ethanolic extract.

Various formulations of CP extracts were examined (a) in *in vitro* skin penetration experiments using Franz diffusion cells, and (b) *in vivo* using the tape-stripping method. Hispidulin penetrated through the skin within 3 hours; vanillic acid and nepetin were absorbed after 6 hours. In contrast, verbascoside was only taken up into the superficial layers of SC. There was no difference in the permeation of hispidulin, nepetin and vanillic acid from 10% w/w CP cream and lotion formulations. Hispidulin was percutaneously absorbed through the skin and taken up into the stratum corneum in the greatest amount, followed by vanillic acid and nepetin. It was found that the *in vitro* model was useful for preliminary formulation development, and that the tape-stripping method was robust and effective.

Verbascoside, although a poor penetrant, was well released from the formulations in an *in vitro* release test, suggesting that it might be a potential skin surface-active compound, such as an antimicrobial. Hispidulin, nepetin and vanillic acid, based on their uptake and penetration into the skin, together with their known biological activities, may be considered as feasible candidates for the development of novel and effective antimicrobial, anti-inflammatory, and antioxidant formulations.

List of abbreviations

A.	*Acanthus*
AE	*Acanthus ebracteatus* Vahl
AFB_1	Aflatoxin B_1
ANOVA	Analysis of variance
APCI	Atmospheric pressure chemical ionization
AP-1-DNA	Activator protein-1-deoxyribonucleic acid
AUC	Area under the curve
B	Baseline correction factor for non-linear model
BHT	Butylated hydroxytoluene
C	Concentration
C.	*Clerodendrum*
CATTM	Centre of Applied Thai Traditional Medicine
CC	Column chromatography
CD	Concentration required to double specific activity
C_d	Solubility of the compound in the homologous formulation
$CDCl_3$	Deuterated-chloroform
CD_3OD	Deuterated-methanol
CMC	Critical micelle concentration
COOH	Carboxylic acid
corr	Corrected
COX	Cyclooxygenase
CO_2	Carbon dioxide
CP	*Clerodendrum petasites* S. Moore
$C_{sat,SC}$	Saturation solubility of the compound in the SC
$C_{sat,W}$	Saturation solubility of the compound in water
C_{SC}	Concentration of the compound in the SC

C_t	Total concentration of dissolved and undissolved compound in the formulation
C_v	Concentration of the compound in the vehicle
CV	Cell viability
C_0	Concentration of the compound in the donor compartment
D	Diffusivity of the compound across the skin
Da	Dalton
DPPH	2,2-diphenyl-1-picrylhydrazyl
D_2O	Deuterium oxide
E.	*Escherichia*
EBV	*Epstein-Barr* virus
EC_{50}	Half maximum effective concentration
ESI	Electrospray ionization
exp	Experimental
FDA	Food and Drug Administration
$FeCl_3$	Ethanolic ferric (III) chloride
GMS-SE	Glycerol monostearate-self emulsifier
HCl	Hydrochloride
HDF	Human dermal fibroblast cell
HPLC	High performance liquid chromatography
HPTLC	High performance thin layer chromatography
H_2SO_4	Sulphuric acid
ICAM	Intercellular adhesion molecule
IC_{50}	Half maximal inhibitory concentration
ICH_{50}	Concentration inhibiting haemolysis by 50%
IE	Inhibitory effect
IL	Interleukin
IPM	Isopropyl myristate
IVRT	*In vitro* release test
J	Transdermal flux

J_{max}	Maximum flux
k_p	Permeability coefficient
$K_{O,W}$	Oil-water partition coefficient
$K_{SC,V}$	SC-vehicle partition coefficient
$K_{SC,W}$	SC-water partition coefficient
L	Diffusion pathlength across the skin
LC_{50}	Median lethal concentration
LD_{50}	Median lethal dose
LOD	Limit of detection
LOQ	Limit of quantification
LPS	lipopolysaccharide
LTB_4	Leukotriene B_4
m	*Meta*
M	Cumulative permeated mass per unit area
MBC	Minimum bactericidal concentration
MIC	Minimum inhibitory concentration
MRSA	Methicillin-resistant *Straphylococcus aureus*
MS	Mass spectrometry
MW	Molecular weight
m/z	Mass to charge ratio
NF-κB	Nuclear factor-kappa B cells
NMF	Natural moisturizing factor
NMR	Nuclear magnetic resonance spectroscopy
NO	Nitric oxide
NRCT	National Research Council of Thailand
o	Oil
o	*Ortho*
OCH_3	Methoxy
OH	Hydroxy

$ONOO^-$	Peroxynitrite
p	*Para*
P	Octanol-water partition coefficient
PDA	Photodiode array detector
ppm	Part per million
pred	Predicted
r^2	Square of the correlation coefficient
RH	Relative humidity
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
RSD	Relative standard deviation
S.	*Straphylococcus*
SC	Stratum corneum
SD	Standard deviation
SFE	Supercritical fluid extraction
SLS	Sodium lauryl sulphate
S/N	Signal-to-noise ratio
Span 60	Sorbitan monooctadecanoate
ss	Steady state
t	Time
TEA	Triethanolamine
TEAC	Trolox equivalent antioxidant activity
TEWL	Transepidermal water loss
$TEWL_0$	Initial transepidermal water loss measured before tape stripping
t_{lag}	Lag time
TLC	Thin layer chromatography
TNF	Tumour necrosis factor
TPA	Tetradecanoylphorbol acetate
t_R	Retention time
Tris	Tris (hydroxymethyl) aminomethane

TS	Tape stripping
Tween 60	Polyethylene glycol sorbitan monostearate
UV	Ultra violet
VEGF	Vascular endothelial growth factor
w	Water
WHO	World Health Organization
x	Cumulative SC thickness removed by tape stripping
δ	Chemical shift
λ	Wavelength
λ_{max}	Maximum wavelength

Chapter 1 Introduction

1.1 Introduction and ethnomedical uses

1.1.1 *Acanthus ebracteatus* Vahl.

A. ebracteatus belongs to the family Acanthaceae and has the English common name Holly Mangrove. The plant is called Ngueak-Plaa-Mo in Thailand and is distributed in the middle, northern, north-eastern, and southern parts of the country. *A. ebracteatus* is a spiny and shrubby herb with dark green spiny leaves which are similar to the leaves of holly (genus *Ilex*). It can grow up to 1.5 metres tall and has purple or white flowers. The fruit is a square-shaped capsule and the seed is off-white and flat. The origin of name *Acanthus* is derived from the Greek "*Akanthos*" meaning "thorn plant" (*Acanthus*: Ngueak-Plaa-Mo, 2009).

Fig. 1.1 *Acanthus ebracteatus* Vahl.

In Thai traditional medicines, the plant is generally prescribed as a single-herb treatment. The whole plant with white flowers has been more commonly used than the purple ones, although there is no difference in terms of clinical efficacy. Medical indications of the plant are broad, such as fever, dizziness, leucorrhoea, arthritis, constipation, asthma, diabetes, tuberculosis, abnormal menstruation, tendon malfunction, kidney stones and as a general agent to promote longevity (Kanchanapoom *et al.*, 2001; Laupattarakasem *et al.*, 2003; Panthong *et al.*, 1986; Sea Holly, 2001; Thangthaisong *et al.*, 2011; Tungjitjaruen, 1977) (S. Tungjitaruen, pers. comm., 2008). Powders, pills and decoctions are usually prepared for oral administration. For topical use, *A. ebracteatus* is traditionally prepared as a poultice (some recipes use the ground herb with coconut oil or sesame oil) and applied to the body to treat skin diseases, including rash, abscess, chronic wounds and snakebites (Hokputsa *et*

al., 2004; Tungjitjaruen, 1977) (S. Tungjitaruen, pers. comm., 2008). The poultice is also recommended to relieve bruising (Hokputsa *et al.*, 2004), pain from an inflamed joint (Panthong *et al.*, 1986) and haemorrhoids (Tungjitjaruen, 1977). The application of juice from the plant is suggested for hair root nourishment (S. Tungjitaruen, pers. comm., 2008).

A. ebracteatus is not only widely used in Thailand, but also in many countries around Southeast Asia. For example, leaf extracts are used to relieve rheumatism in India (Uses of mangrove plant products: Traditional uses of mangrove plant products in India, 1998). Seeds are regarded as remedies for cough and boils (Guide to the mangroves of Singapore, 2001; Hokputsa *et al.*, 2004), and the juice of the leaves are prescribed to prevent hair loss in Malay folklore (Sea Holly, 2001).

1.1.2 *Clerodendrum petasites* S. Moore.

C. petasites (English name: One Root Plant) is in the family Lamiaceae according to The International Plant Names Index (IPNI) (*Clerodendrum petasites* S. Moore, 2005). The Latin name "*Clerodendrum*" was first given by Linnaeus in 1753 and was changed to the Greek word "*Clerodendron*" by Adanson in 1763. The Greek form "*Klero*" means chance or destiny and "*dendron*" means tree and thus the generic name which may derive from ancient belief. For example, some species bring or do not bring good luck (e.g., *Clerodendron fortunatum* and *C. infortunatum*); some had healing properties, whereas others had opposite reactions. The Latin name "*Clerodendrum*" was revived by Moldenke in 1942 and is used in modern taxonomy (Hsiao and Lin, 1995; Rueda, 1993; Shrivastava and Patel, 2007).

There are numerous Thai names from each region, for instance, Ping-Khom and Ping-Luang in the north, Phaya-Rak-Deaw in the south, Nang-Shon and Phom-Phee in the northeast. However, Thao-Yaai-Mom from the midland is the best known. The plant is widespread in the middle, north-eastern, and southern parts of Thailand. It is an erect shrub with dark green opposite or whorled leaves. The height is 1-2 metres and the flower is white with a cup-shaped calyx, typically with 5 lobes. The fruit is rounded and black when ripe (Klaiklay, 2009; Singharachai *et al.*, 2011a; Thao-Yai-Mom).

Fig. 1.2 *Clerodendrum petasites* S. Moore.

Thai traditional practitioners usually prepare aerial parts, leaves, or roots of *C. petasites* as a tea, alcoholic extract or cigarette to treat asthma (Hazekamp *et al.*, 2001; Panthong *et al.*, 2003; Panthong *et al.*, 1986). Leaves and roots are also ground into powders for treatment of inflammation (Panthong *et al.*, 1986) as well as to treat fever, cough, and vomiting (Panthong *et al.*, 2003; Thai traditional medical textbook: Paet-Ta-Ya-Saat-Song-Kror (แพทยศาสตร์สงเคราะห์), 2007) (S. Tungjitaruen, pers. comm., 2011). The plant is widely prescribed for oral administration and generally formulated into multi-herb recipes. The most famous recipe is “Ha-Rak” (synonyms: Ben-Cha-Lo-Ka-Wi-Chian, Kaew-Ha-Dueng, Phed-Sa-Wang), containing equal amounts by weight of five roots from *C. petasites*, *Ficus racemosa* Linn, *Capparis micracantha* DC, *Harrisonia perforate* Merr, and *Tiliacora triandra* Diels (Pichaensoonthon *et al.*, 2005). The recipe is currently registered by the Thai Food and Drug Administration (FDA) for antipyretic activity (List of herbal medicinal products, 2006; National list of essential medicines: Ha-Rak). Dosage forms of Ha-Rak are powders, tablets and capsules, but decoction is conventionally served. There are fewer records for topical remedies. Poultices are most often formulated for skin diseases, such as rash, abscess, urticaria, snakebites and insect bites (Panthong *et al.*, 2003; Pongboonrot, 1965; Thai traditional medical textbook: Paet-Ta-Ya-Saat-Song-Kror (แพทยศาสตร์สงเคราะห์), 2007) (T. Tipcharoentham, pers. comm., 2011; S. Tungjitaruen, pers. comm., 2011). Many recipes are dispersed in alcohol, especially Thai rice whisky, before application. It is notable that *C.*

petasites is prescribed to treat rash which has not resulted from bacterial or fungal infection, while *A. ebracteatus* is more commonly used for skin infection.

C. petasites is also widely distributed in many other countries, e.g., Malaysia, India, Southern China, Sri Lanka, and Vietnam. Ethnomedical uses of the plant are found in their medical systems. For example, root and leaf extracts of *C. petasites* have been documented for the treatment of rheumatism, asthma and other inflammatory diseases (Shrivastava and Patel, 2007). In India, fruits are reportedly used to reduce fertility in males and the plant is used to cure malaria in China (Hazekamp *et al.*, 2001; Panthong *et al.*, 2003; Shrivastava and Patel, 2007).

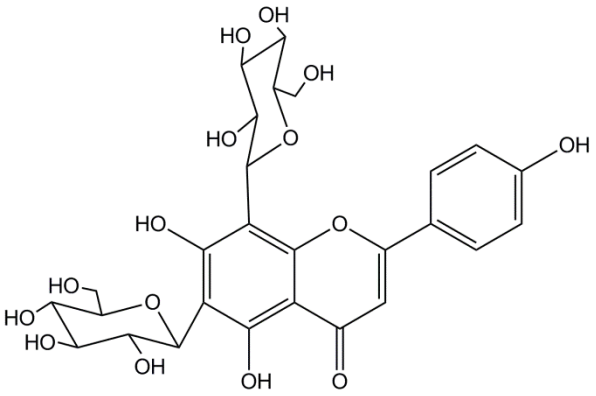
1.2 Phytochemistry in *A. ebracteatus*

Chemical constituents and polysaccharides of *A. ebracteatus* have been investigated. Twenty seven compounds (five new and twenty two known compounds presented in Table 1.1) were isolated and identified from the aerial part of the plant by Kanchanapoom *et al.* (2001). The chemical structures have been reviewed by Li *et al.* (2009) and are shown in Fig. 1.3 to Fig. 1.10.

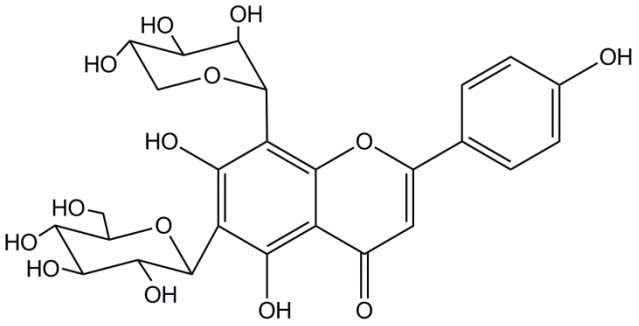
Table 1.1 Natural chemicals identified in *A. ebracteatus*.

Categories	**Compounds**
Flavonoids	Vecenin-2, Schaftoside, Luteolin-7-*O*-β-D-glucuronide, Apigenin-7-*O*-β-D-glucuronide
Phenylpropanoids	Verbascoside (acteoside), Isoverbascoside (isoacteoside), β-Hydroxyacteoside, Cistanoside E, Leucosceptoside A, Martynoside
Lignans	(+)-Lyoniresinol 3α-*O*-β-D-glucopyranoside, (-)-Lyoniresinol 3α-*O*-β-D-glucopyranoside, (8*R*,7'*S*,8'*R*)-5,5'-Dimethoxylariciresinol 4'-*O*-β-D-glucopyranoside, (+)-Syringaresinol-4-*O*-β-D-apiofuranosyl-(1→2)-*O*-β-D-glucopyranoside, Magnolenin C
Megastigmane glycosides	Plucheoside B, Alangionoside C, Ebracteatoside A, Premnaionoside
Aliphatic alcohol glycosides	Ebracteatoside B, Ebracteatoside C, Ebracteatoside D
Benzoxazinoid glycosides	(2*R*)-2-*O*-β-D-Glucopyranosyl-2H-1, 4-benzoxazin-3(4H)-one (HBOA-Glc, blepharin), (2*R*)-2-*O*-β-D-Glucopyranosyl-4-hydroxy-2H-1,4-benzoxazin-3(4H)-one (DIBOA-Glc), 7-Chloro-(2*R*)-2-*O*-β-D-glucopyranosyl-4-hydroxy-2H-1,4-benzoxazin-3(4H)-one (7-Cl-DIBOA-Glc)
Phenylethanol glycosides	2-Phenylethyl 8-*O*-β-D-glucopyranosyl-(1→2)-*O*-β-D-glucopyranoside, Benzyl alcohol 7-*O*-β-D-glucopyranosyl-(1→2)-*O*-β-D-glucopyranoside (zizybeoside I)
Nucleoside	Adenosine

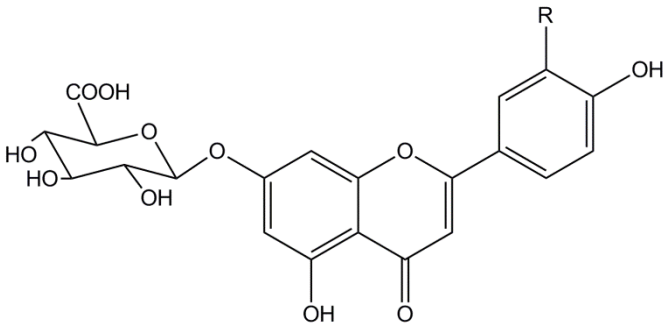
Hokputsa *et al.* (2004) also studied bioactive water-soluble polysaccharides from *A. ebracteatus* stems by anion-exchange chromatography. The plant neutral and acidic fractions were rich in galactose, 3-*O*-methylgalactose, arabinose, galacturonic acid, and rhamnose.



Vecenin-2

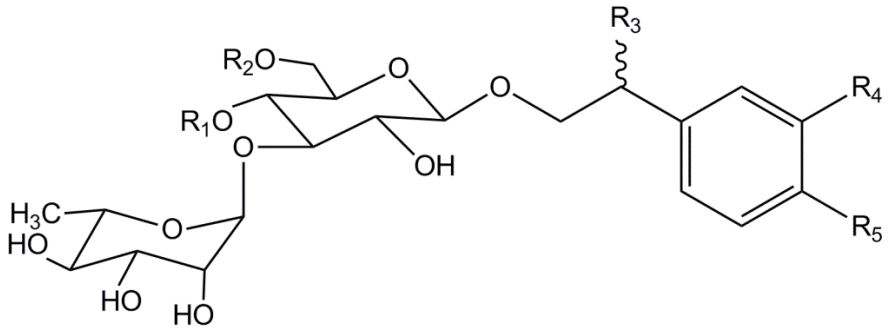


Schaftoside

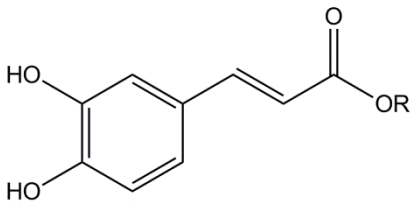


	R
Luteolin-7-*O*-β-D-glucuronide:	OH
Apigenin-7-*O*-β-D-glucuronide:	H

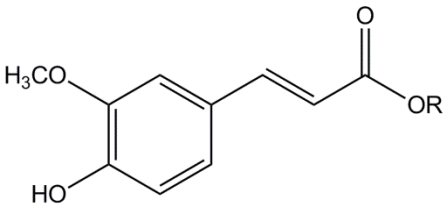
Fig. 1.3 Flavonoids in *A. ebracteatus.*



	R_1	R_2	R_3	R_4	R_5
Verbascoside:	*E*-Caffeoyl	H	H	OH	OH
Isoverbascoside:	H	*E*-Caffeoyl	H	OH	OH
β-Hydroxyacteoside:	*E*-Caffeoyl	H	OH	H	H
Cistanoside E:	H	H	H	OH	OH
Leucosceptoside A:	*E*-Feruloyl	H	H	OH	OH
Martynoside:	*E*-Feruloyl	H	H	OMe	OH

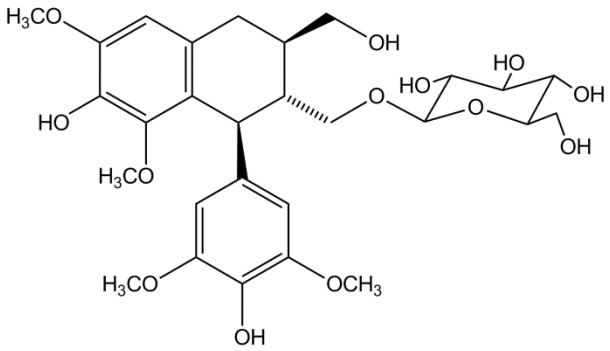


Caffeoyl

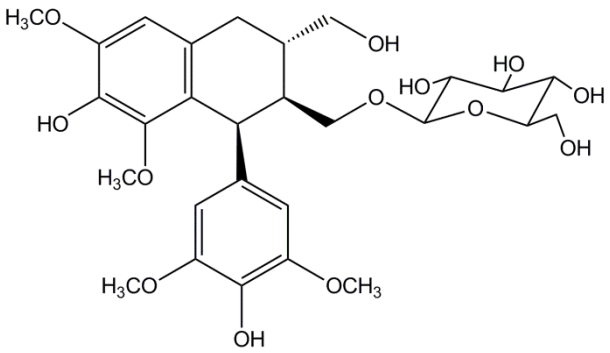


Feruloyl

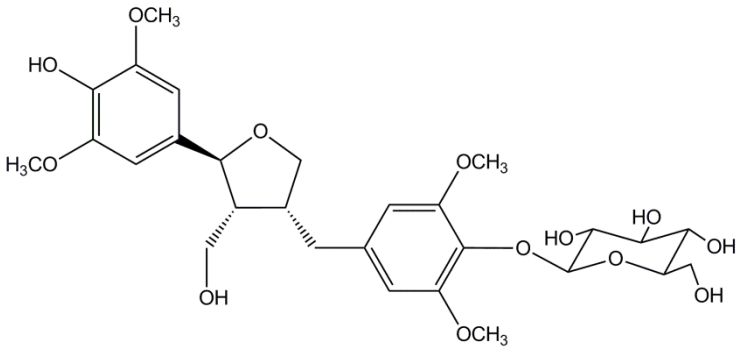
Fig. 1.4 Phenylpropanoids in *A. ebracteatus.*



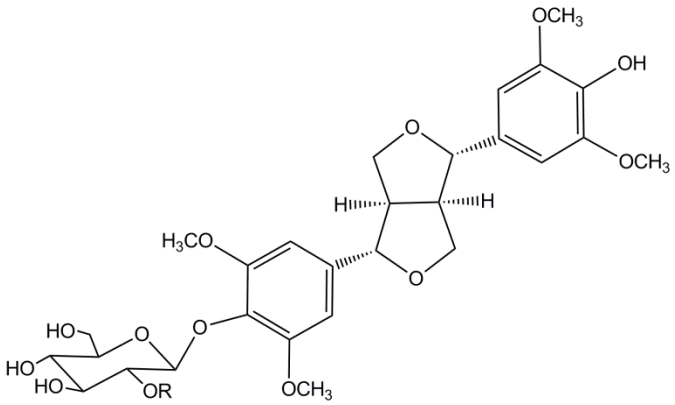
(+)-Lyoniresinol 3α-*O*-β-D-glucopyranoside



(-)-Lyoniresinol 3α-*O*-β-D-glucopyranoside

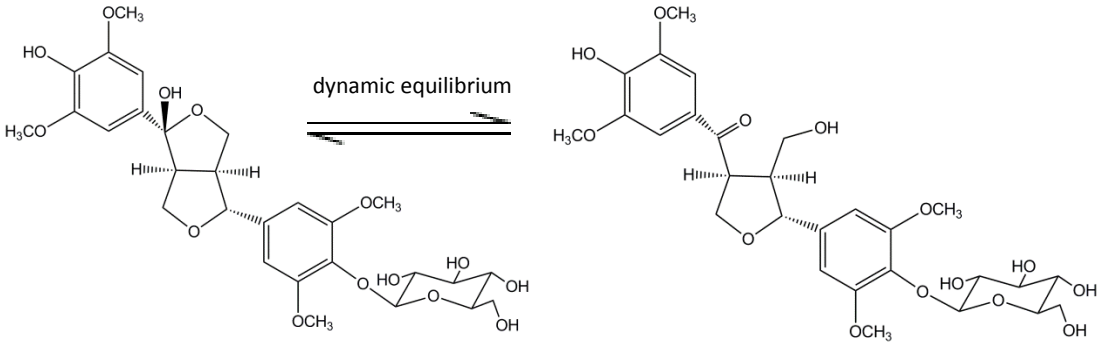


(8*R*,7'*S*,8'*R*)-5,5'-Dimethoxylariciresinol 4'-*O*-β-D-glucopyranoside



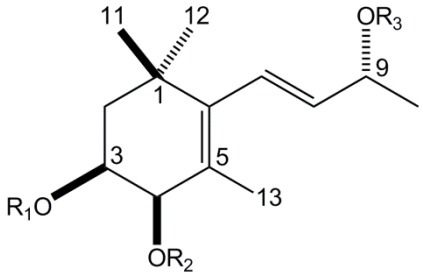
(+)-Syringaresinol-4-*O*-β-D-apiofuranosyl-(1→2)-*O*-β-D-glucopyranoside:

R = β-D-apiofuranosyl

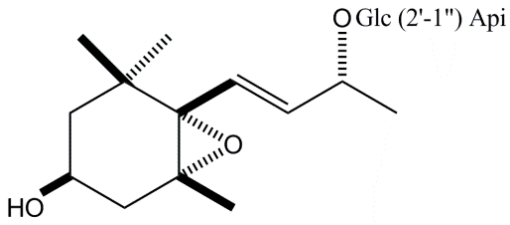


Magnolenin C

Fig. 1.5 Lignans in *A. ebracteatus.*

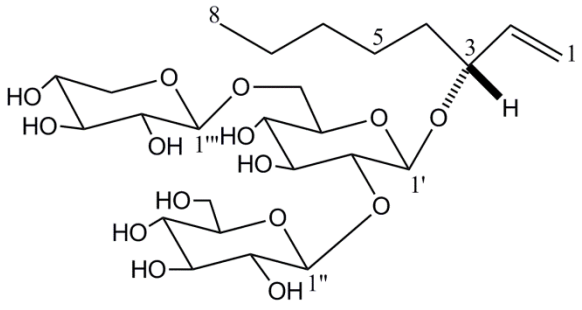


	R_1	R_2	R_3
Plucheoside B:	Glc	H	H
Alanginoside C:	H	Glc	H
Ebracteatoside A:	H	H	Glc (2’-1”) Api

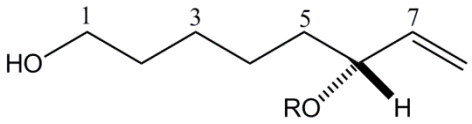


Premnaionoside

Fig. 1.6 Megastigmane glycosides in *A. ebracteatus.*

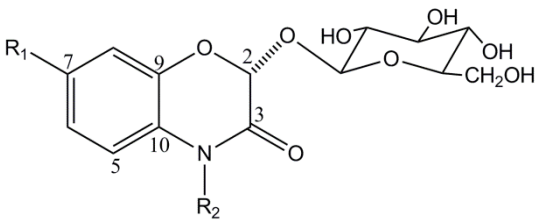


Ebracteatoside B



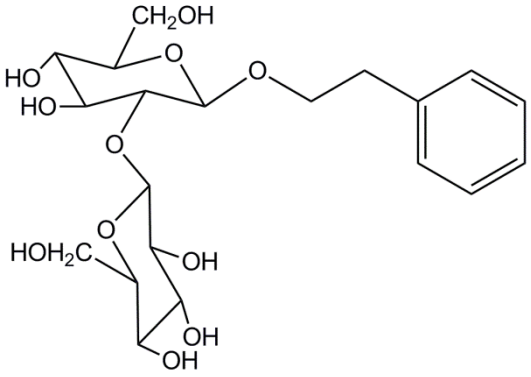
	R
Ebracteatoside C:	Glc (2’-1”) Glc
Ebracteatoside D:	Glc (6’-1”) Xyl

Fig. 1.7 Aliphatic alcohol glycosides in *A. ebracteatus.*

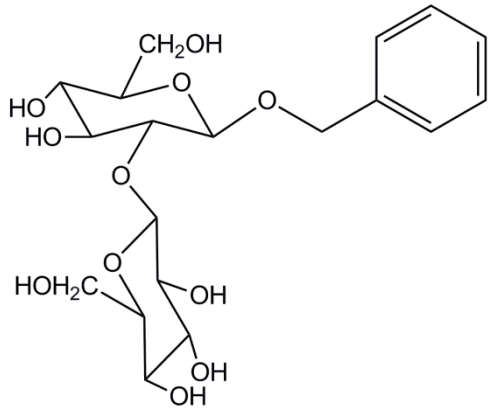


	R_1	R_2
HBOA-Glc:	H	H
DIBOA-Glc:	H	OH
7-Cl-DIBOA-Glc:	Cl	OH

Fig. 1.8 Benzoxazinoid glycosides in *A. ebracteatus.*

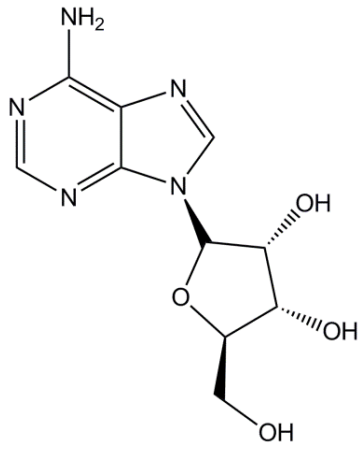


2-Phenylethyl 8-*O*-β-D-Glucopyranosyl-(1→2)-*O*-β-D-glucopyranoside



Benzyl 7-O-β-D-glucopyranosyl-(1→2)-*O*-β-D-glucopyranoside

Fig. 1.9 Phenylethanol glycosides in *A. ebracteatus.*



Adenosine

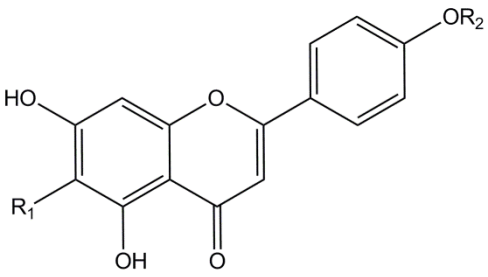
Fig. 1.10 Nucleoside in *A. ebracteatus.*

1.3 Phytochemistry in *C. petasites*

Although the chemical constituents in genus *Clerodendrum* have been widely investigated, there have been only a few studies on *C. petasites*. The compounds found in the aerial parts and roots of *C. petasites* are shown in Table 1.2 (Hazekamp *et al.*, 2001; Klaiklay, 2009; Singharachai *et al.*, 2011a; Thongchai *et al.*, 2007) and the chemical structures are displayed in Fig. 1.11 to Fig. 1.14.

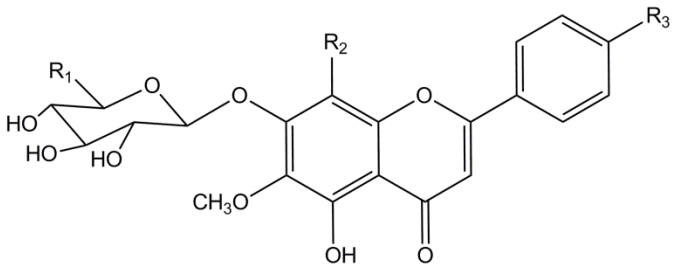
Table 1.2 Natural compounds identified in *C. petasites*.

Categories	**Compounds**
Flavones	Apigenin, Hispidulin, 6,4'-Dimethoxyscutellarin
Flavone glycosides	Hispidulin 7-methylglucuronide, Nevadensin 7-glucoside
Glycoside	Arbutin
Diterpenoid	Bungene A



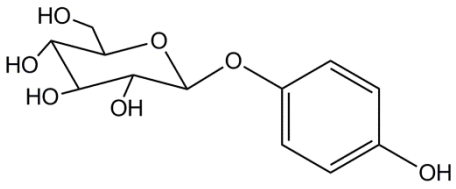
	R_1	R_2
Apigenin:	H	H
Hispidulin:	OCH_3	H
6,4'-Dimethoxyscutellarin:	OCH_3	CH_3

Fig. 1.11 Flavones in *C. petasites*



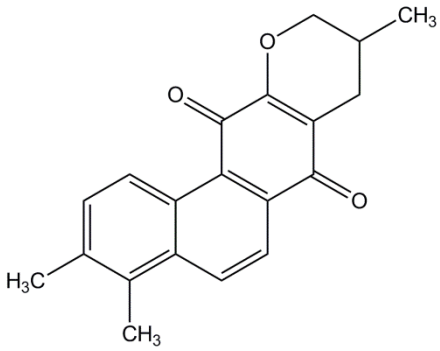
	R_1	R_2	R_3
Hispidulin 7-methylglucuronide:	$COOCH_3$	H	OH
Nevadensin 7-glucoside:	CH_2OH	OCH_3	OCH_3

Fig. 1.12 Flavone glycosides in *C. petasites.*



Arbutin

Fig. 1.13 Glycoside in *C. petasites.*



Bungene A

Fig. 1.14 Diterpenoid in *C. petasites.*

1.4 Biological activities

1.4.1 Biological activities of compounds in *A. ebracteatus*

The inhibition of mutagenesis by organic extracts of the stems of *A. ebracteatus* has been reported by Rojanapo *et al.* (1990). Both purified (by column and thin layer chromatographies) and partially purified extracts (50-500 µg) inhibited indirect mutagens (aflatoxin B_1, AFB_1) toward *Salmonella typhimurium* TA100 via the activity of aniline hydroxylase which is one of the cytochrome P450 drug-metabolizing enzymes.

Murakami *et al.* (1994) found that methanol extracts (20 $\mu g \cdot mL^{-1}$) from leaves of *A. ebracteatus* show moderate inhibition of tumour promotion (70% > Inhibitory effect; IE ≥ 50%) in the *Epstein-Barr* virus (EBV) activation test and low toxicity (cell viability; CV > 70%) in the cytotoxicity test. Bunyapraphatsara *et al.* (2003) noted that leaves from the plant also possess cancer chemoprevention activity by the reduction of electrophilic quinones by quinone reductase (concentration required to double specific activity; CD = 9.5 $\mu g \cdot mL^{-1}$, and 50% inhibition of cell growth; IC_{50} = 17.6 $\mu g \cdot mL^{-1}$). Later, Mahasiripanth *et al.* (2012) reported supportive data that aqueous extracts of *A. ebracteatus* inhibited angiogenesis in a cervical cancer-derived cell line (CaSki) in mice with the half maximal inhibitory concentration (IC_{50}) of 5 $mg \cdot mL^{-1}$ after incubation for 48 hours. The study also showed that reduction of vascular endothelial growth factor (VEGF) was achieved when treating mice with oral high dose of the plant extracts (3 g/kg body weight/day) for both 14- and 28-day experiment periods. It was therefore suggested that the plant extracts could inhibit cervical cancer growth. It is also worth noting that there was no significant difference in angiogenesis results between the aqueous plant extracts and controls in human dermal fibroblast cells (HDFs).

Antioxidant and lipid peroxidation activities of *A. ebracteatus* leaves were reported by Bunyapraphatsara *et al.* (2003). Half maximum effective concentration (EC_{50}) in free radical scavenging was 49 $\mu g \cdot mL^{-1}$ and IC_{50} in lipid peroxidation inhibition was 29 $\mu g \cdot mL^{-1}$.

Ethanolic and aqueous extracts from leaves of *A. ebracteatus* possess anti-inflammatory activity by inhibiting eicosanoid synthesis (Laupattarakasem *et al.*, 2003). The reduction in leukotriene B_4 (LTB_4) production was 64% and 44%, respectively, in the ethanolic and water extracts at 500 $\mu g \cdot mL^{-1}$.

A 3-*O*-methyl galactan-rich fraction isolated from stems of *A. ebracteatus* was highly active (concentration inhibiting haemolysis by 50%; ICH_{50} = 10 $\mu g \cdot mL^{-1}$) in the complement fixation

test, either activation or inhibition in the immune system (Hokputsa *et al.*, 2004). Koschella *et al.* (2008) then synthesized methyl galactan in order to achieve the biological activity, however, the synthetic polysaccharide was relatively less potent. The concentrations of the test samples were up to 500 $\mu g \cdot mL^{-1}$.

Aqueous extracts of *A. ebracteatus* possess antibacterial activity by inhibition of nosocomial skin infection pathogens and skin infection bacteria including gram positive bacteria: *Staphylococcus aureus*, *S. epidermidis*, and *Lactobacillus plantarum*, and gram negative bacteria: *Klebsiella pneumonia*, and *Proteus vulgaris* (Sittiwet et al., 2009). Minimum inhibitory concentrations (MICs) and minimum bactericidal concentrations (MBCs) were reported in the range of 1-2 and 2-4 $g \cdot L^{-1}$, respectively, and were compared to those of gentamicin sulphate, a renowned antibiotic, which were less than 0.5 $mg \cdot L^{-1}$ for MICs and were not determined for MBCs.

Wound healing effects of ethanolic extracts from ground stems of *A. ebracteatus* combined with a collagen scaffold (a tissue-engineered skin substitute) were studied by Somchaichana *et al.* (2012). The combination significantly promoted wound closure by increasing angiogenesis in the proliferative phase when compared to control groups (individual application of normal saline, the plant extracts and the collagen scaffold) after 14 days in a mouse model. The plant dried extracts mixed with normal saline, 0.3 g/kg body weight, was topically applied on a wound area of approximately 2.25 cm^2 (1.5 x 1.5 cm).

1.4.2 Biological activities of compounds in *C. petasites*

Ethanolic extracts from aerial parts of *C. petasites* possess spasmolytic activity on guinea-pig tracheal smooth muscle and this activity has been attributed to the flavonoid hispidulin (Hazekamp et al., 2001). The crude extracts had an EC_{50} value of about 4.8 $mg \cdot mL^{-1}$ which was much weaker than aminophylline (EC_{50} = 26 $\mu g \cdot mL^{-1}$), the well-known drug for the treatment of asthma. However, pure hispidulin was more potent, with an EC_{50} about 3.0 (± 0.8) x 10^{-5} M, equivalent to 9 $\mu g \cdot mL^{-1}$.

Methanolic extracts of *C. petasites* have moderate anti-inflammatory and potent anti-pyretic effects caused by the inhibition of prostaglandin synthesis (EC_{50} = 2.3 mg/ear and 420 mg/kg in rats) without ulcerogenic effect (Panthong *et al.*, 2003). The plant extracts were also fairly nontoxic with acute toxicity LD_{50} (the median lethal dose) of 5000 mg/kg.

The antioxidant arbutin, a notably effective whitening agent, was identified and quantified as 1.5 μg/g in the aqueous extracts from roots of *C. petasites* by high performance liquid chromatography (HPLC) (Thongchai *et al.*, 2007).

Singharachai *et al.* (2011b) reported that aqueous extracts from the roots of *C. petasites* have weak radical scavenging activity (IC_{50} > 1 mg·mL^{-1}) and moderate anti-mutagenicity (40-60% inhibition). However, the ethanolic extracts exhibited antioxidant activity in the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical assay at IC_{50} = 249 μg·mL^{-1}. Both extracts presented the median lethal concentration (LC_{50}) above 10 mg·mL^{-1}; in other words, no cytotoxicity.

1.5 Topical and transdermal drug delivery

1.5.1 Structure and function of the skin

Skin is the largest organ of the body accounting for around 10% of the body mass and has a surface area of 1.7 m^2 on average. It keeps "the insides in and the outside out" (Williams, 2003) by preventing excessive heat and water loss from the body and protecting the body from substances in the external environment. The skin, shown in Fig. 1.15, consists of three main layers (from the outermost to the innermost): epidermis, dermis, and hypodermis.

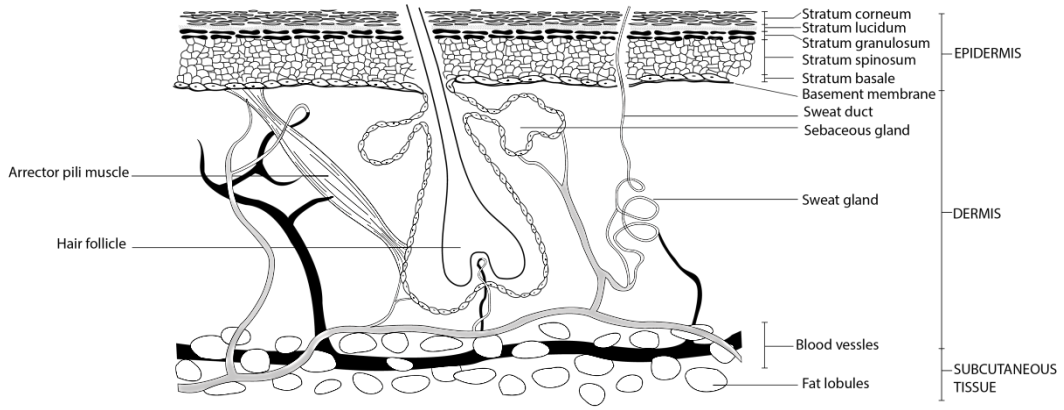


Fig. 1.15 Cross-section of the skin (adapted from Williams (2003)).

The epidermis is a multiply layered membrane from 0.06-0.8 mm thick depending on the part of the body. With no blood vessels, diffusion is the principal means of molecular transport in the epidermis (e.g., nutrients and waste products). The stratified squamous

epithelium is histologically classified into five distinct layers (from the outside to the inside) comprising stratum corneum (SC), stratum lucidum, stratum granulosum, stratum spinosum, and stratum basale.

The SC (horny layer) is around 10 µm thick when dry and contains terminally differentiated keratinocytes (corneocytes) embedded in lipid bilayers. Corneocytes contain high levels of protein (predominantly keratin) (Wilkes *et al.*, 1973) and are separated by multiple lipid bilayers comprised of ceramides, fatty acid, cholesterol, cholesterol sulphate and sterol/wax esters (Elias, 1983; Wertz *et al.*, 1985). Michaels *et al.* (1975) and Elias (1981) introduced a 'brick and mortar' model for the SC by representing corneocytes as bricks and lipid lamellae as mortar (Fig. 1.16). Corneodesmosomes penetrate through the lipid bilayers and create a proteinaceous cellular junction between corneocytes that play a key role in mechanical protection. Natural moisturizing factor (NMF) composed of low molecular weight compounds (especially amino acids) retains water in the SC to maintain flexibility and overall structural integrity.

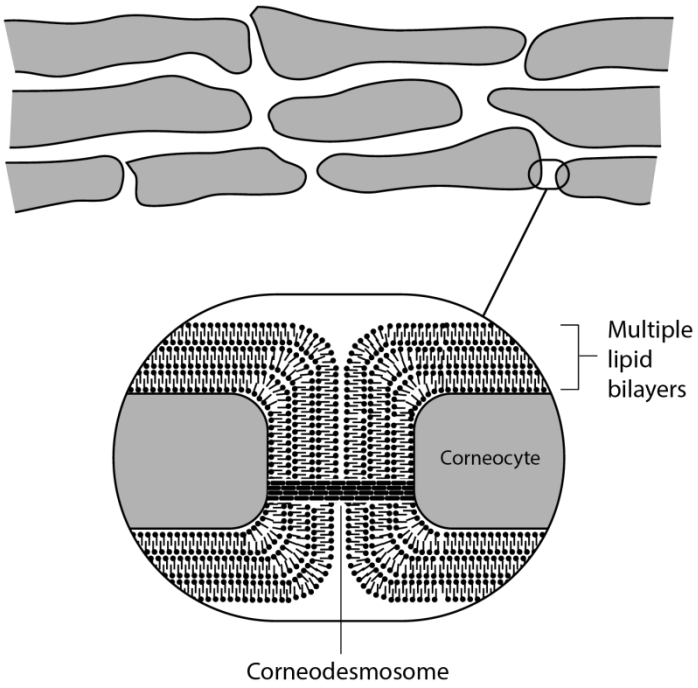


Fig. 1.16 A 'brick and mortar' model (adapted from Williams (2003)).

The layers between the SC and the dermis are known as the viable epidermis. The stratum basale (stratum germinativum, basal layer) mainly generates keratinocytes. Keratinocytes at the lowest layer are connected to the dermo-epidermal membrane (basement membrane, a membrane to separate the epidermis and the dermis) by strong proteinaceous cellular bridges called hemidesmosomes. Melanocytes, Langerhans cells and Merkel cells are also located in the basal layer and are bound to keratinocytes by dendritic linkages. Melanocytes produce the pigment melanin relevant to the skin colour, ultra violet (UV) absorption and free-radical scavenging. Langerhans cells are antigen-presenting cells and are the outermost elements of the immune system preventing invasion of the body by foreign organisms. Merkel cells are relevant to cutaneous sensation, particularly light-touch discrimination. Only keratinocytes are differentiated upward through stratum spinosum, stratum granulosum, stratum lucidum to the SC and during which morphological changes to polygonal, flat, and anucleate cells take place.

The dermis (corium) provides flexibility and support to the skin. It is around 3-5 mm thick and contains connective and elastic tissues, collagen fibrils, blood and lymphatic vessels, nerve endings, hair follicles, sebaceous and sweat glands, embedded in gelatinous mucopolysaccharides (Wilkes *et al.*, 1973). The vasculature in the dermis plays many essential roles, such as body temperature control, nutrient delivery, and waste removal of small particles (Cross and Roberts, 1993). The lymphatic system is also important in the elimination of large molecules (Cross and Roberts, 1993), immunological response, and interstitial pressure regulation. Sebum produced by the sebaceous gland maintains skin hydration and flexibility. Additionally, sebum and a dilute salt solution (secreted by the sweat gland) provide a pH of around 5 on the skin surface which is beneficial to the adhesion of resident skin microflora to the skin (Lambers *et al.*, 2006). Normal skin flora together with the acidic pH on the skin surface prevents colonization of pathogenic bacteria leading to skin infections and inflammatory responses (Ki and Rotstein, 2008; Schmid-Wendtner and Korting, 2006). The acidity of the SC is also crucial to homeostasis of the cutaneous permeability barrier via the generation of ceramides involving two lipid processing enzymes: β-glucocerebrosidase (βGlcCer'ase) and acidic sphingomyelinase (Hachem *et al.*, 2003; Holleran *et al.*, 1992; Uchida *et al.*, 2000). Furthermore, the normal skin pH retains SC integrity and cohesion and presents the premature dissolution of corneodesmosomes (Fluhr *et al.*, 2001; Hachem *et al.*, 2003).

The hypodermis (subcutaneous fat layer) is comparatively thick and is composed of abundant adipose tissues to maintain body heat and protect against mechanical stress.

With respect to topical and transdermal drug delivery studies, the lipophilic SC is the most important barrier. The dermis, however, is hydrophilic and can serve as a barrier for very lipophilic compounds. Clearance of penetrating drugs from the skin is via resorption into the dermal microcirculation and lymphatic system.

1.5.2 Routes of transdermal delivery

Compounds can penetrate into or through the skin via three possible pathways: transappendageal, transcellular, and intercellular routes (Fig. 1.17), utilising different features of skin anatomy.

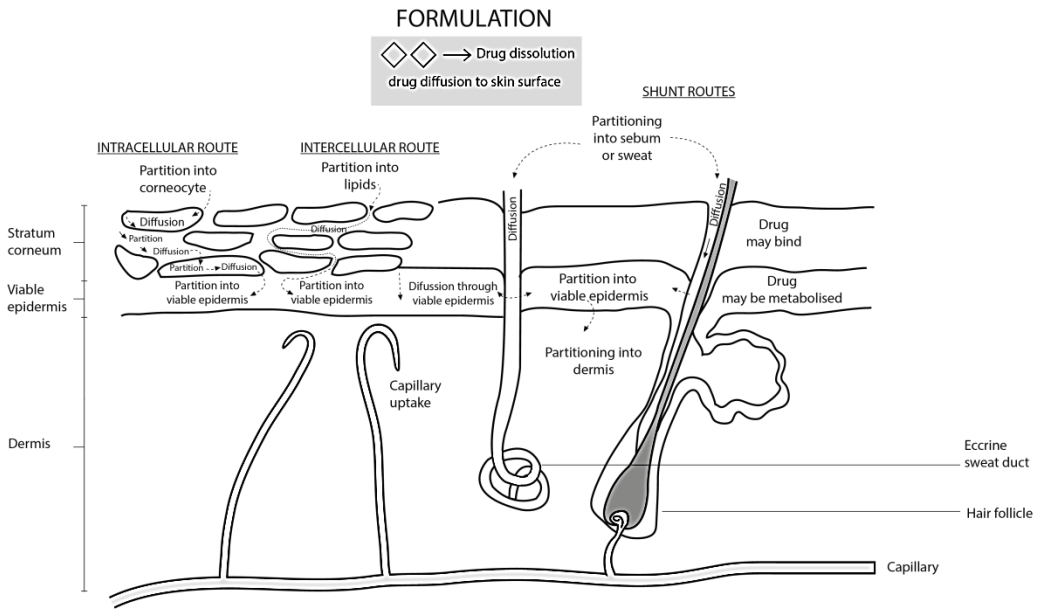


Fig. 1.17 Permeation pathways (redrawn from Williams (2003)).

Transappendageal transport (shunt route) offers compounds the least resistance pathway through the hair follicles and sweat ducts. Although the initial penetration rate is rapid and dominated by this pathway, this route makes a less important contribution to the total flux across human skin because the appendages offer a very limited surface area available for transport.

Transcellular (intracellular) pathway requires molecules to repeatedly partition between and diffuse through the corneocytes and multiple intercellular lipid bilayers between them. Corneocytes are less permeable than the lipid domains (Heisig *et al.*, 1996) and this route is therefore considered to be of low importance.

Intercellular pathway involves a continuous route through the multiple lipid bilayers, which offer, at least in theory, both polar and non-polar environments (Brian and Walters, 1993). This pathway is generally accepted as the predominant transdermal route for small molecules (Albery and Hadgraft, 1979; Boddé *et al.*, 1989).

1.5.3 Cutaneous metabolism

Regardless of the route transdermal passage, penetrating molecules may be metabolised by epidermal enzymes. Although a key advantage of topical and transdermal drug delivery is to avoid first-pass metabolism in the liver, there remains a possibility that drugs can be metabolised in the skin (Williams, 2003). Skin metabolism is typically less than 10% of that in the liver (Hotchkiss, 1998). Possible reactions are oxidation, reduction, hydrolysis, methylation and glucuronidation. Hydrolysis by esterases is the main activity in the skin. *Staphylococcus epidermidis* – a normal skin flora – may also metabolise applied drugs.

1.5.4 Prediction of drug penetration through intact skin

In general, the chemical constituents of natural products possess a wide range of physicochemical properties and not all are able to penetrate through the skin. Absorption is related to many factors including molecular weight (MW), lipophilicity, and solubility in oil and in water; physiological factors such as skin age, body site, and race; skin environment (e.g., temperature and humidity); duration and area of exposure; and the applied formulations and the excipient therein.

Of the factors influencing the compound's absorption, the physicochemical properties are the most important. A good chemical candidate for topical and transdermal delivery should have an intermediate octanol-water partition coefficient (log P of 1-3 (Hadgraft and Somers, 1956)), small size (MW of 100-500 Dalton, Da), and be unionised. In addition, low melting point compounds tend to be better absorbed (Ghafourian *et al.*, 2010).

Fick's first law of diffusion (Eq. 1.1) can be used to estimate the maximum possible flux (J_{max}; amount per unit area per unit time) of a chemical across the skin:

$$J_{max} = \frac{D}{L} \cdot C_{sat,SC} \qquad \text{Eq. 1.1}$$

where D is the diffusivity of chemical across, typically, the SC (units: $cm^2 \cdot h^{-1}$); L is the diffusion pathlength through this membrane (length); $C_{sat,SC}$ is the saturation solubility of the compound in the SC (amount per unit volume).

Guy (2010) presented the theoretical development of Fick's first law to predict the J_{max} of fragrance chemicals. To do so, the saturation solubility of the compound in water ($C_{sat,W}$) replaces $C_{sat,SC}$ in the equation via the simple relation in Eq. 1.2 to yield a rewritten form of Fick's first law (Eq. 1.3):

$$K_{SC,W} = \frac{C_{sat,SC}}{C_{sat,W}} \qquad \text{Eq. 1.2}$$

$$J_{max} = \frac{D}{L} \cdot K_{SC,W} \cdot C_{sat,W} \qquad \text{Eq. 1.3}$$

where $K_{sc,w}$ is the SC-water partition coefficient of the compound (no units). The permeability coefficient of the compound (k_p; units: $cm \cdot h^{-1}$) is defined in Eq. 1.4 allowing further simplification to Eq. 1.5.

$$k_p = \frac{D \cdot K_{SC,W}}{L} \qquad \text{Eq. 1.4}$$

$$J_{max} = k_p \cdot C_{sat,W} \qquad \text{Eq. 1.5}$$

The empirical equation of Potts and Guy (1992) expressed in Eq. 1.6 permits k_p values ($cm \cdot h^{-1}$) to be estimated.

$$\log k_p = -2.72 + 0.71 \cdot \log P - 0.0061 \cdot MW \qquad \text{Eq. 1.6}$$

where P is the octanol-water partition coefficient of the compound (no units) and MW is its molecular weight (Dalton, Da).

With respect to highly lipophilic compounds (generally, log P > 3), the rate of permeation will be affected by the viable epidermis (viewed as a hydrophilic layer) as these molecules partition out of the SC slowly. In these situations, a correction to the k_p calculated from Eq. 1.6 must be made (Cleek and Bunge, 1993), and the modified value (k_p^{corr}) used in Eq. 1.5 to estimate J_{max}:

$$k_p^{corr} = \frac{k_p}{1 + \frac{k_p \cdot \sqrt{MW}}{2.6}} \qquad \text{Eq. 1.7}$$

It follows that values of MW, log P and $C_{sat,W}$ are required to calculate J_{max} (via Eqs. 1.5, 1.6 and 1.7). The MW is normally known. Where experimental values of the latter two parameters are not available, they can be obtained using (for example) the ALOGPS 2.1 algorithm from the Virtual Computational Chemistry Laboratory and/or from ChemSpider (ALOGPS 2.1 algorithm, 2001; Chemspider).

1.5.5 Assessment of topical and transdermal delivery

Topical and transdermal drug delivery are of considerable interest for treating both local and systemic diseases. However, in both cases, the skin's outermost layer, the SC, represents a formidable barrier to drug penetration. To improve topical drug administration, and to develop suitable formulations, it is therefore necessary to have available useful *in vitro* and *in vivo* methods with which to determine and to quantify the rate and extent of percutaneous absorption.

Although *in vivo* experiments are most relevant, they are the more difficult to perform and analyse. Therefore, the use of *in vitro* models is common while recognising that extrapolation of results to the *in vivo* situation may be complex.

With respect to mathematical models of skin permeation, Fick's first law and Fick's second law of diffusion have been applied respectively to infinite and finite dosing. Fick's first law of diffusion states that the rate of movement of the molecule is proportional to the concentration gradient as shown in Eq. 1.8.

$$J = -D \cdot \frac{\partial C}{\partial x} \qquad \text{Eq. 1.8}$$

where J is the diffusion rate; D is diffusivity of compound; $\partial C/\partial x$ is the concentration gradient (C is the concentration and x is the space coordinate measured normal to the direction of transport).

When diffusion is assumed to be unidirectional (one-dimensional diffusion from the outside to the inside of the skin), Fick's second law is derived (Eq. 1.9) and expresses the concentration (C) of the permeating solute as a function of both time (t) and position (x).

$$\frac{\partial C}{\partial t} = D \cdot \frac{\partial^2 C}{\partial x^2} \qquad \text{Eq. 1.9}$$

The equation is valid when the SC is assumed to be a pseudo-homogeneous membrane and D is independent of time and position. Several equations for different application dose conditions have been developed based on Fickian diffusion (Higuchi, 1961; Kushner *et al.*, 2007; McCarley and Bunge, 1998, 2000; Reddy *et al.*, 1998).

1.5.5.1 Infinite dosing (pseudo-steady-state permeation)

The model applies when the drug concentration on the skin surface remains essentially constant and depletion is not significant over the experimental period. In other words, the donor concentration should not decrease by more than 10% from the original value (Williams, 2003). Under infinite dosing conditions, Fick's second law of diffusion can be simplified and derived for *in vitro* and *in vivo* permeation studies.

1.5.5.1.1 Infinite dosing: *in vitro* model

The *in vitro* experiments comprise a donor compartment and a receiver, separated by an excised piece of skin. The donor chamber is filled with the formulation from which the penetrant diffuses through the skin and into the receptor solution underneath. Franz diffusion cells are most commonly used (Fig. 1.18).

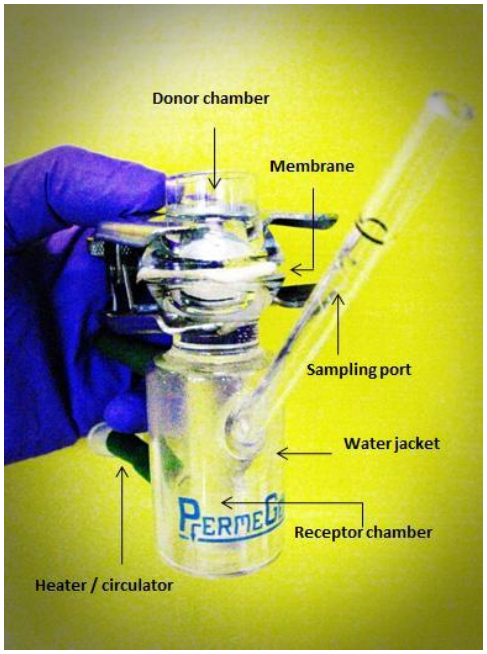


Fig. 1.18 Franz diffusion cell.

It is essential that the receptor solution provides sink conditions (zero concentration at the beginning and less than 10% of the penetrant's saturated concentration throughout the time of the experiment (Topical and transdermal drug products, 2009)). The excised piece of skin is assumed to be a homogeneous barrier with no binding between the skin components and the permeant molecule. It is also assumed that the formulation does not alter the nature of the skin. Fick's second law of diffusion (Eq. 1.9) then simplifies at steady state to:

$$\frac{dM}{dt} = D \cdot \frac{C_{SC}}{L} \qquad \text{Eq. 1.10}$$

where M is the cumulative mass of permeant passing into the receiver per unit area of the skin in time, t; D is the diffusivity of compound across the skin; C_{SC} is the concentration of the permeant in the first layer of the skin (in contact with the formulation); L is the pathlength for diffusion across the skin.

Applying the same theoretical development described in section 1.5.4 (Eq. 1.1 -1.5), Eq. 1.10 reduces to:

$$\frac{dM}{dt} = J = k_p \cdot C_v \qquad \text{Eq. 1.11}$$

where J is the transdermal flux; k_p is the permeability coefficient of the compound; C_v is the concentration of the permeant in the vehicle (donor solution).

The permeation profile following an infinite dose application can be displayed as the cumulative amount of drug per unit area as a function of time (Fig. 1.19). The pseudo-steady-state flux (J_{ss}) is obtained from the linear slope. The extrapolation from the pseudo-steady-state line to the x-intercept expresses the lag time (t_{lag}) which is defined as (Crank, 1975):

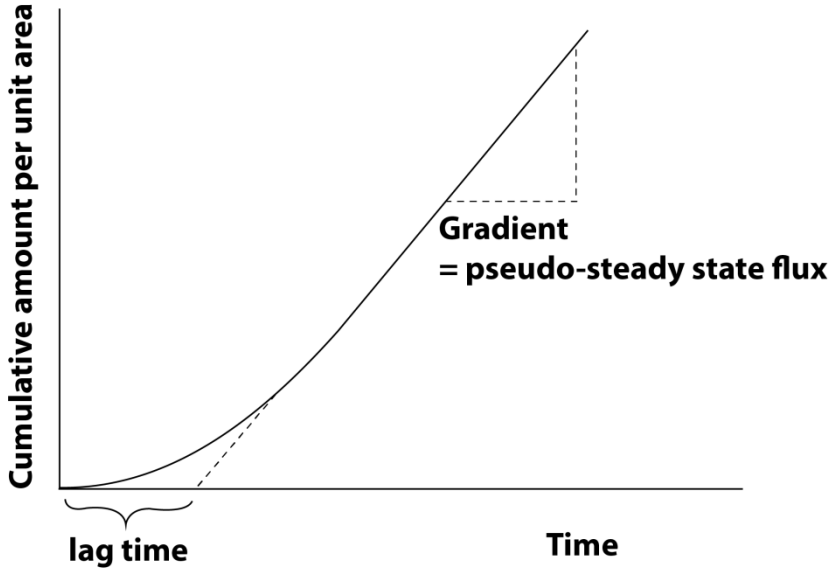


Fig. 1.19 The cumulative amount of drug penetrating through the SC per unit area as a function of time under infinite dose conditions (redrawn from Williams (2003)).

$$t_{lag} = \frac{L^2}{6D} \qquad \text{Eq. 1.12}$$

To reach steady state transport, experiments should be conducted for approximately three lag times (Shah, 1993).

However, before reaching steady state (Scheuplein, 1967), the cumulative permeated mass per unit area (M_t) is given by the following solution to Fick's second law:

$$M_t = K_{SC,V} \cdot L \cdot C_0 \cdot \left[\frac{D \cdot t}{L^2} - \frac{1}{6} - \frac{2}{\pi^2}\sum_{n=1}^{\infty}\frac{(-1)^n}{n^2}\exp\left(-Dn^2\pi^2\frac{t}{L^2}\right)\right] \quad \text{Eq. 1.13}$$

where $K_{sc,v}$ is the partition coefficient between membrane and vehicle; L is the effective diffusion pathlength; C_0 is the essentially constant concentration of the permeant in the donor compartment.

At steady state, Eq. 1.13 reduces to:

$$M_{t,ss} = J_{ss} \cdot \left(t - t_{lag}\right) \quad \text{Eq. 1.14}$$

where

$$J_{ss} = C_0 \cdot \frac{K_{SC,V} \cdot D}{L} \quad \text{Eq. 1.15}$$

$$k_p = \frac{J_{ss}}{C_0} = \frac{K_{SC,V} \cdot D}{L} \quad \text{Eq. 1.16}$$

and $M_{t,ss}$ is the cumulative permeated mass per unit area at steady-state; J_{ss} is the steady–state flux.

1.5.5.1.2 Infinite dosing: *in vivo* model

For *in vivo* studies, the skin is also viewed as a homogeneous membrane. Using tape-stripping, Fick's second law can again be solved to provide an expression for the concentration profile across the SC as a function of time and position (Herkenne *et al.*, 2007):

$$C(x,t) = K_{SC,V} \cdot C_0 \cdot \left[\left(1 - \frac{x}{L}\right) - \frac{2}{\pi}\sum_{n=1}^{\infty}\frac{1}{n}\sin\left(\frac{n\pi x}{L}\right)\exp\left(-\frac{Dn^2\pi^2 t}{L^2}\right)\right] \quad \text{Eq. 1.17}$$

where x is the relative position inside the barrier with $0 \le x \le L$.

This equation is valid when: (1) substance's concentration at the skin surface (C_0) is constant; (2) sink conditions are provided at x = L; (3) SC is completely empty of drug at t = 0. It has been suggested that if the time required to completely remove SC by tape stripping (t_{TS}) is less than 20% of

lag time (t_{lag}), and that the duration of drug exposure (t_{exp}) is more than 0.3 t_{lag}, then the results from tape stripping will not be influenced by the time required in tape-stripping (Reddy et al., 2002). An example concentration-depth profile is presented in Fig. 1.20.

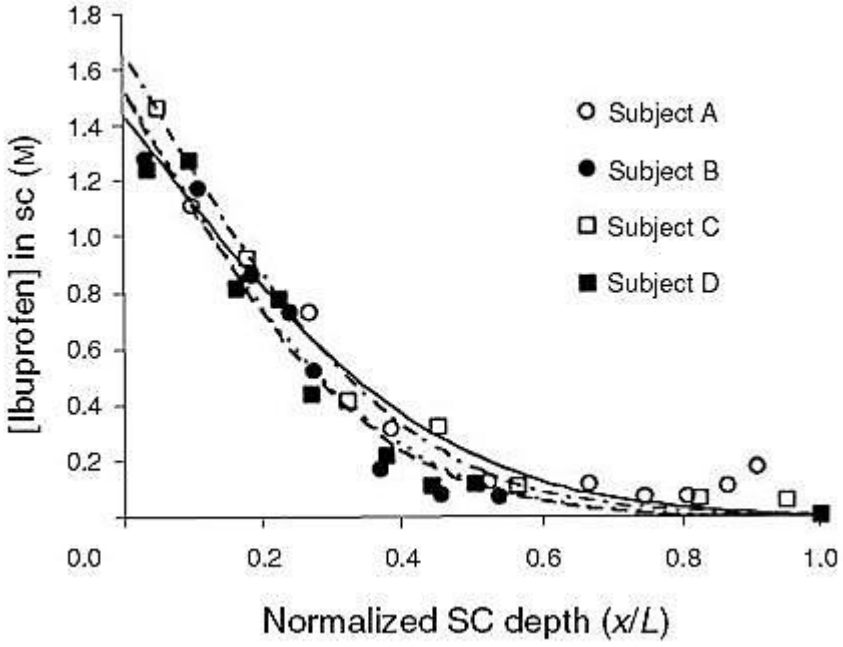


Fig. 1.20 The concentration of drug in the SC profile as function of the relative SC thickness (example graph from Herkenne *et al.* (2007)).

1.5.5.2 Finite dosing (transient permeation)

The cumulative absorption shows a marked time dependency due to significant drug depletion over the experimental period (Mitragotri *et al.*, 2011). Thus, while the flux initially increases, it then reaches a maximum value and subsequently declines as the available drug remaining to diffuse from the applied phase decreases towards zero.

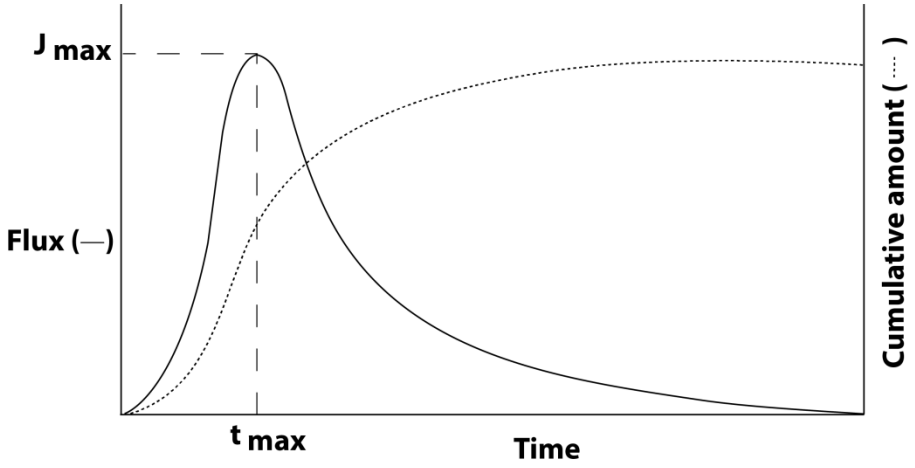


Fig. 1.21 Permeation profile following a finite dose application (redrawn from Williams (2003)).

In general, the most frequently used experimental approach is infinite dosing where the donor concentration is assumed to remain constant and not to deplete during the experiment. The pseudo-steady-state flux is obtained as discussed in section 1.5.5.1. However, the amount of drug applied depends on the specific study design. Thus, in the laboratory setting, it is not unusual to use a saturated solution or a suspension of the penetrant. However, clinically with the practical use of topical products (e.g., creams, lotions, and gels), finite dosing is common.

1.5.6 Topical formulations

Dermatological formulations are developed to produce a local effect in the skin and typically result in very low systemic drug levels. While the latter are nonetheless rather easy to quantify with today's sophisticated analytical tools, the determination of drug levels in the skin (and in particular at the specific site of action) remains a significant challenge. Indeed, for very few dermatological drugs is the therapeutic dose within the skin actually known.

A successful formulation for their treatment has to balance the need to provide a sufficient driving force for drug diffusion, and to ensure efficacy with the provision of a stable product and the absence or minimisation of any adverse effects. This leads to a conundrum in Fick's first law.

$$J = \left(\frac{D}{L}\right) \cdot K_{SC,V} \cdot C_V \qquad \text{Eq. 1.18}$$

The diffusivity (D) of drug across the SC and the pathlength for diffusion (L) are not parameters that can be manipulated very much by changing a formulation. This means that the flux (J) is directly dependent upon the product of the drug's SC-vehicle partition coefficient ($K_{SC,V}$) and its concentration in the vehicle (C_V). Drug stability is certainly flavoured by its high solubility in the vehicle (i.e., a formulation in which the drug is "content") but this means that its "leaving tendency" is low and partitioning into the SC somewhat unfavoured. In contrast, if the drug does not like the vehicle and has poor solubility therein, the loading one can achieve is reduced making it perhaps difficult to sustain delivery for a decent period of time. There is some compensation here, however, afforded by the fact that partitioning out of the vehicle (i.e., the leaving tendency) will be greater.

Thus, a successful formulation must find a balance between these competing features by striving for a system in which the drug is present at, or very close to, saturation in the residual film left in the skin post-application and in which the drug remains sufficiently soluble to sustain delivery for the required treatment period. Topical dosage forms commonly found on the market are pastes, ointments, creams and lotions.

1.5.6.1 Pastes

Pastes are semisolid dosage forms containing solid drug dispersed in a single aqueous phase with high polymer content or in an oleaginous (fatty) base, typically a mixture of soft paraffin, hard paraffin and polyethylene. Pastes are very similar to ointments but are stickier, harder and less greasy, and can contain up to 50% solids in the total formulation weight.

Pastes are particularly good for absorbing fluid; for example, seropus from wounds and noxious chemicals from bacteria. A thick and unbroken film of a paste is also useful for skin protection from sun and wind burn (Barry, 1988). Pastes are not recommended for application to hairy areas as they are difficult to remove by washing.

1.5.6.2 Solutions

Solutions are transparent liquid preparations containing at least one compound (solute) completely dissolved in the vehicle. The latter typically comprises a single solvent or a co-solvent (solubilizer) mixture, such as alcohol or glycerol, to increase drug solubility. Other excipients can be added but they must be miscible with the vehicle, e.g., preservatives, stabilizers, and colorants.

Most solutions are prepared in an aqueous base and leave a thin film on the skin which is not ideal for a protective effect. However, they are useful to treat wet lesions, to apply to hairy areas, and to promote rapid dermal absorption.

1.5.6.3 Emulsions (creams and lotions)

Emulsions are semisolid preparations of two immiscible liquids stabilised by emulsifiers. One liquid makes up the internal phase which is dispersed in the other (the continuous phase). As emulsions are also colloids, they are able to scatter light. When the droplets are large, the colloid scatters long wavelength (low frequency) light and the emulsion has a yellow colour. Reducing the droplet size by vigorous mixing or homogenisation causes the emulsion to appear progressively whiter as the light is being scattered equally at all wavelengths. If the droplets are even smaller (≤ 0.05 μm), light will transmit through the emulsion without being scattered and result in transparency as found in microemulsions.

1.5.6.3.1 Types of emulsions

There are four main types of emulsions: oil in water (o/w), water in oil (invert emulsion, w/o), multiple emulsions ($w_1/o/w_1$, $w_1/o/w_2$, $o_1/w/o_1$, $o_1/w/o_2$), and microemulsions (transparent emulsions).

Oil in water emulsions (o/w) have small oil droplets dispersed in the external phase of water (Fig. 1.22). This emulsion is the most commonly used for pharmaceutical and cosmetic purposes.

Water in oil emulsions (w/o) contain small aqueous globules dispersed in oil which is the continuous phase (Fig. 1.22). This emulsion is greasier and more difficult to prepare than an o/w emulsion.

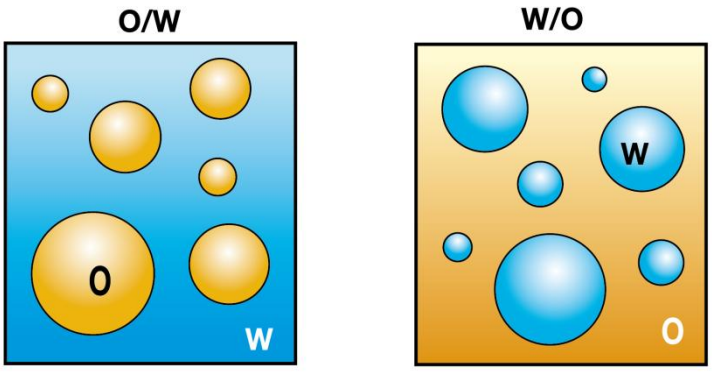


Fig. 1.22 Normal or simple emulsions: (a) oil in water emulsion; (b) water in oil emulsion.

Multiple emulsions are complex systems. In this case, the dispersed phase contains a simple emulsion (o/w or w/o) (Fig. 1.23). The primary emulsion is stabilised by appropriate amounts of surfactants; hydrophilic for o/w and lipophilic for w/o. Multiple emulsions can be divided into 2 major groups: water-in-oil-in-water (w/o/w) and oil-in-water-in-oil (o/w/o). The water phase in w/o/w emulsion can be the same or different (i.e., $w_1/o/w_1$, $w_1/o/w_2$), and likewise for the oil phase in o/w/o ($o_1/w/o_1$, $o_1/w/o_2$). These multiple emulsions can be very useful for controlled drug release (Okochi and Nakano, 2000), improving the stability of sensitive compounds (Lee *et al.*, 2004), and increasing skin penetrability (Rajesh *et al.*, 2012).

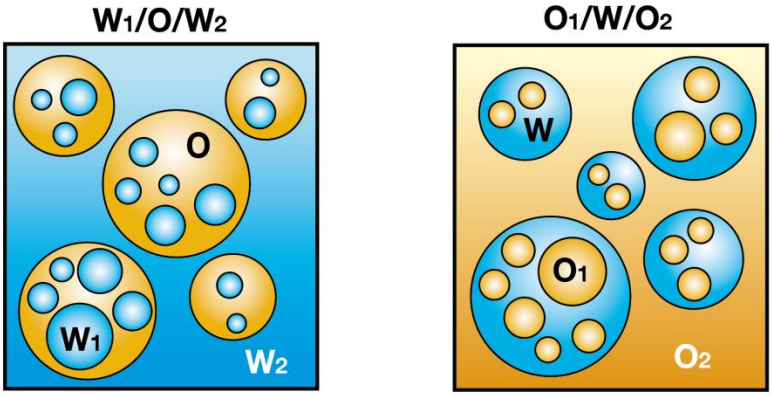


Fig. 1.23 Multiple emulsions: (a) water-in-oil-in-water (w/o/w) emulsion; (b) oil-in-water-in-oil (o/w/o) emulsion.

Microemulsions are transparent formulations containing very small droplets (5-100 nm) in the internal phase. More than one type of surfactant in high amount is used in their preparation so that the product is stable. Microemulsions are mostly used in the cosmetics field.

1.5.6.3.2 Emulsifiers (intermediate phase, emulsifying agent, emulgent)

An emulsifier is a substance which stabilises two immiscible liquids in an emulsion. Emulsifying agents are categorized as primary (true) and auxiliary (quasi, secondary).

The primary emulsifier aids emulsification by reducing surface interfacial tension between the internal and the external phases, or/and generating a repulsive force after being adsorbed around the dispersed droplets, or/and creating films surrounding the internal droplets which are sterically stabilised. These mechanisms cause the internal droplets to remain suspended and not aggregate. In some cases, emulsions can be stabilised with the primary emulsifier alone without adding the auxiliary. The most well-known emulsifiers of this group are the synthetic surfactants, such as sodium lauryl sulphate (SLS), the Tweens (ether esters), and the Spans (sorbitan esters).

The secondary emulsifier increases the viscosity in the dispersion medium thereby decreasing the chance that the globules in the internal phase can aggregate. These secondary emulsifiers cannot be used alone because they cannot stabilize the suspension enough. Examples of secondary emulsifiers are tragacanth, agar, gelatin and cholesterol.

It is worth noting that some emulsifiers possess both primary and secondary properties, such as methylcellulose and sodium carboxymethylcellulose.

1.5.6.3.3 Surfactants (surface active agents)

Surfactants are synthetic compounds consisting of hydrophilic (polar) and hydrophobic (non-polar) segments. They are very versatile in function, acting as detergents in cleansing processes, as emulsifying agents, as wetting and suspending agents, as solubilising agents, and as absorption enhancers. Surfactants can be classified by structure and ionization into anionic, cationic, amphoteric (zwitterion) and non-ionic. Cationic surfactants, mainly quaternary ammonium compounds (e.g., benzalkonium chloride), possess potent antibacterial activity; zwitterionic surfactants are less potent (Ishikawa *et al.*, 2002; Merianos, 1991). Cationic surfactants also cause more skin irritation and cytotoxicity than anionic and non-ionic surfactants (Effendy and Maibach, 1996; Hall-Manning *et al.*, 1998; Harvell *et al.*, 1994; Lansdown and Grasso, 1972).

A common property of surfactants is that they form micelles. At low concentration, surfactants disperse individually in the external phase as monomers. With increasing concentration, surfactants form a surface monolayer decreasing the interfacial surface tension. Once the surface is covered, addition of further surfactant leads to aggregation of these molecules in the bulk phase and the formation of micelles (Fig. 1.24) at the critical micelle concentration (CMC). At the CMC, the surface tension decreases no further and remains constant (Fig. 1.25).

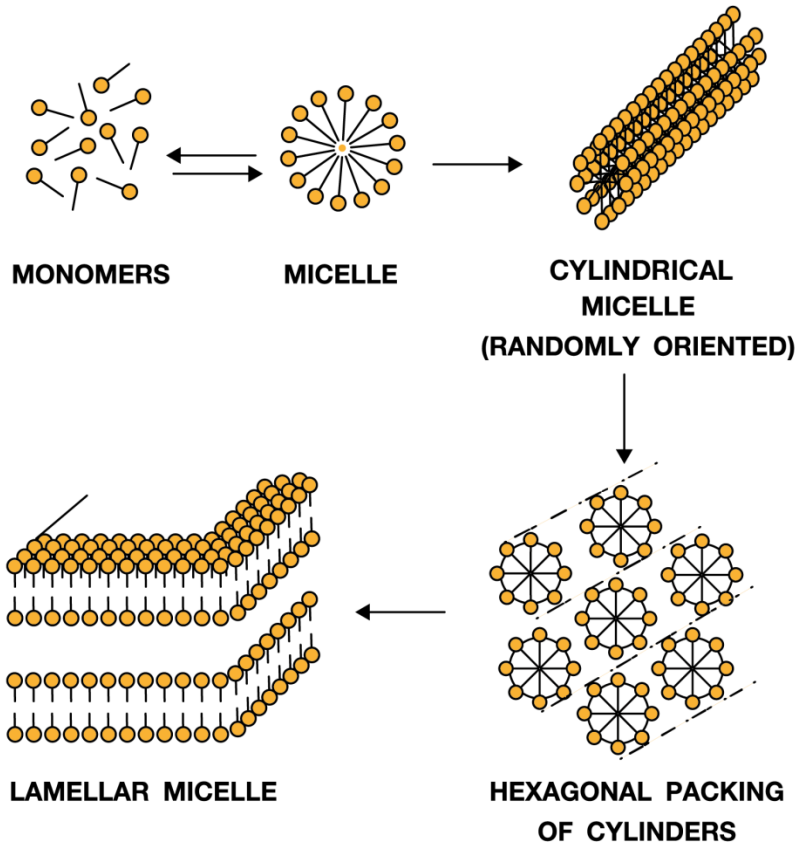


Fig. 1.24 Illustration of the micellar formation when increasing surfactant concentration (Shah *et al.*, 1977).

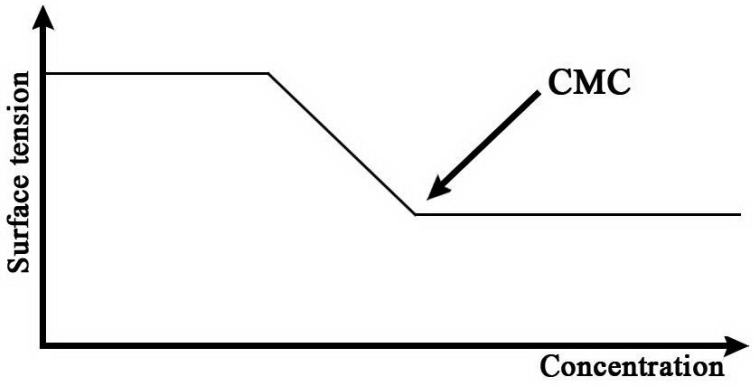


Fig. 1.25 Surface-tension concentration curve.

Surfactant micelles are spherical at the CMC with cylindrical, hexagonal and lamellar micellar structures appearing as the amount of surfactant in the continuous phase increases (Fig. 1.24).

1.6 Thesis purposes

Acanthus ebracteatus Vahl and *Clerodendrum petasites* S. Moore are Thai medicinal plants that have been widely used in Thai traditional medicines for many indications including skin complaints. Much of the knowledge and recipes available in Thai traditional medicine are written in Thai ancient language and transmitted verbally down the generations to keep them secret. Consequently, the information is not readily accessible and is insufficient in scientific background.

The plants have been available in local markets and Thai traditional clinics (e.g., Ayurved clinic, Center of Applied Thai Traditional Medicine, Faculty of Medicine Siriraj Hospital, Mahidol University) for at least thirty years. Even so, only crushed leaves and powders are available on the market with poor reliability in quality and reproducibility with respect to the active ingredients. These forms are also inconvenient to use and transport.

No clinical trials with these plant preparations have been reported, although some biological activities have been studied. In terms of the chemical species identified, numerous natural compounds in *A. ebracteatus* have been discovered; mainly polysaccharides. Unlike *A. ebracteatus*, only seven compounds have been characterised in *C. petasites*. With respect to topical delivery, there is no information concerning the local bioavailability of active species from these plants.

Currently, the National Research Council of Thailand (NRCT) has announced “The eighth national research policy and strategy 2012-2016” to support research work throughout the country. One of the major strategies is to develop and conserve Thai traditional medicines and support research in natural products.

An investigation of these plants from initial identification through to *in vivo* skin permeation experiments with an optimised product will support the ethnomedical uses and establish guidelines for topical delivery studies of natural products.

The aims of this study are:

- To identify and characterize topically active substances in Thai medicinal herbs by qualitative and quantitative analyses using high performance liquid chromatography (HPLC) coupled with mass spectrometry (MS).

- To develop and optimize suitable dosage forms for topical delivery to the skin, concentrating on solutions and emulsions, evaluated by *in vitro* diffusion cell experiments.

- To determine the topical bioavailability of the active substances following delivery to the skin *in vivo* in human volunteers using tape stripping methodology.

Chapter 2 Experimental

2.1 Materials

2.1.1 Plant materials

Dried samples of *A. ebracteatus* and *C. petasites* were authenticated and obtained from the Ayurved Siriraj Manufacturing Unit of Herbal Medicines and Products, Centre of Applied Thai Traditional Medicine (CATTM), Faculty of Medicine Siriraj Hospital, Mahidol University, Thailand. Extracts were produced by maceration using 80% ethanol and by supercritical fluid extraction (SFE) as described in sections 2.1.1.1 and 2.1.1.2 below, and subsequently evaporated to dryness. Five batches of ethanolic extracts of both plants and one batch of SFE extract were kept separately in light protective and airtight containers and stored in a desiccator at room temperature.

The ethanolic extracts were separated into water, butan-1-ol, ethyl acetate and petroleum ether fractions by liquid-liquid partition (procedure 2.1.1.3). Only the butanol and ethyl acetate fractions were further separated by column chromatography as explained in section 2.1.1.4. All the fractions were kept in light protective and airtight containers and stored at 4°C.

2.1.1.1 Ethanolic extraction by maceration

The whole plants were washed twice with water, air-dried for 2 hours, and oven-dried at 50°C for 10 hours. The dried plants were ground and sieved to fine powders having particle size of 425 µm (by US sieve number 40). The powders (1 kg) were weighed, then suspended with 80% ethanol (10 L) and macerated overnight at room temperature. The suspensions were filtered through a paper filter by suction (Whatman Glass microfibre filters (GF/A) 110 mm, Whatman International Ltd., Maidston, UK; GAST model DOA-V517-BN, GAST Manufacturing Inc., USA), and the first filtrates were obtained. The residues were macerated with 80% ethanol again overnight at room temperature before being filtered to yield the second filtrates. Both filtrates were combined, evaporated at 40°C under reduced pressure to remove ethanol, freeze dried at -70°C (Revco Ultimate Plus, Thermo Fisher Scientific

(Asheville) LLC, USA) and subsequently lyophilized to dryness (Alpha 2-4 Isc., Martin Christ Company, Germany).

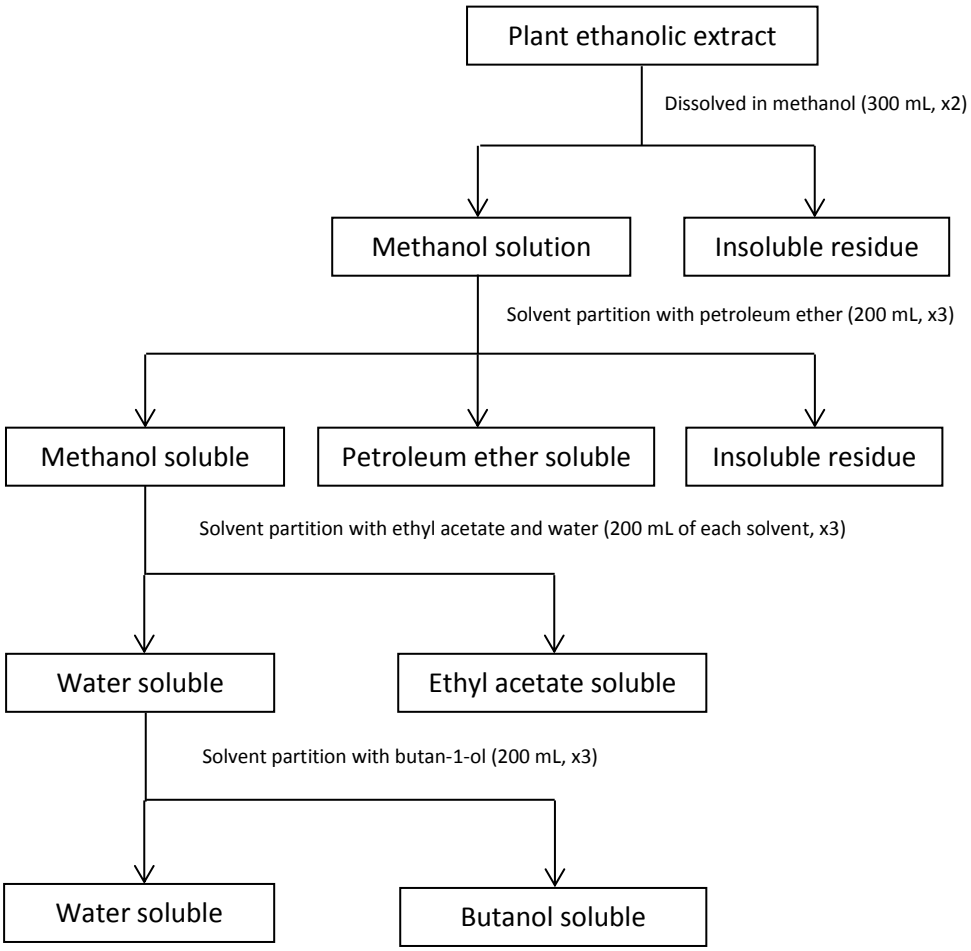
2.1.1.2 Supercritical fluid extraction (SFE)

SFE is a technique to extract compounds of interest by using supercritical fluid, carbon dioxide (CO_2), as an eluent. Ethanol was used as a co-solvent to dissolve the more polar compounds.

The powders of crude plants were weighed (45 g) and placed into a supercritical fluid extractor (PolyScience, USA). The optimised condition was controlled at 60°C at a pressure of 500 bar. Carbon dioxide was delivered at a flow rate of 2 L·min^{-1} and ethanol was diffused at 1 L·min^{-1} for an hour.

2.1.1.3 Liquid-liquid partition

The ethanolic extracts (15.5 g) were separated into water, butan-1-ol, ethyl acetate and petroleum ether fractions by liquid-liquid partitioning (Scheme 2.1). All the fractions were evaporated to dryness in a rotary evaporator (Buchi Rotavapor R-114 with Buchi Waterbath B-480, Switzerland). High performance thin layer chromatography (HPTLC) and nuclear magnetic resonance (NMR) spectroscopy were used to select the highest flavonoid content fraction. Only the butanol fraction was injected into the HPLC-MS and HPLC-PDA when identifying phenolic compounds in the plants.



Scheme 2.1 Procedures of liquid/liquid partition.

2.1.1.4 Column chromatography

A mixture of butanol and ethyl acetate fractions was separated by a preparative chromatography column through 40 g silica gel (0.035-0.007 mm, 60 A, Acros, USA, chromatography grade) eluted by a step gradient of 100% ethyl acetate followed by 1%, 2%, 5%, 10%, 20%, 50% methanol in ethyl acetate and 100% methanol (400 mL of each solvent mixture). Fractions of 40 mL were collected and evaporated to dryness under reduced pressure before being dissolved in methanol for screening by HPTLC and characterization by HPLC-MS.

2.1.2 Chemicals and reagents

Twenty five phenolic compounds, including caffeic acid, 4-coumaric acid, naringin, chrysin, 5,7-dimethoxycoumarin, gallic acid, rosemarinic acid, kaempferol, cinnamic acid, arbutin (Sigma-Aldrich, USA), vanillic acid, ferulic acid, apigenin (Fluka Analytical, China), rutin, quercetin (Koch-Light Laboratories Ltd., UK), verbascoside, naringenin, chrysoeriol, hesperetin, luteolin, diosmetin, nepetin, scutellarein (Extrasynthese, France), hispidulin (Tocris Bioscience, UK), cirsimaritin (BioBioPha. Co; Ltd.), were of analytical grade.

Mobile phases for HPLC-MS and HPLC-PDA consisted of HPLC grade acetronitrile (Fisher Scientific, UK), HPLC grade water obtained from a deionized water treatment system (Millipore, MA, USA) and MS grade acetic acid (Fluka Analytical, Germany). Deuterated-methanol (methanol-D_4, CD_3OD), deuterated-chloroform (chloroform-D, $CDCl_3$) and deuterium oxide (D_2O) were used for NMR analysis and purchased from Cambridge Isotope Laboratories, Inc., UK. Other chemicals and reagents, such as isopropyl myristate (Fluka Analytical, Germany), tris (hydroxymethyl) aminomethane hydrochloride (Tris-HCl, Acros Organics, USA), tris aminomethane (Trizma® base, Sigma-Aldrich, USA), formic acid (Riedel-de Haën, Germany), methanol, ethanol (Sigma-Aldrich, USA), butan-1-ol (Fisher Scientific, UK), ethyl acetate, petroleum ether and ferric (III) chloride, were of analytical grade. Acetone was of laboratory grade.

Excipients of topical formulations comprised propyl paraben (propyl 4-hydroxybenzoate), stearyl alcohol (1-octadecanol), Tween 60 (polyethylene glycol sorbitan monostearate), Span 60 (sorbitan monooctadecanoate), glycerol, mineral oil, and triethanolamine (TEA) from Sigma-Aldrich, USA. Methyl paraben (methyl 4-hydroxybenzoate) and cetyl alcohol were purchased from Fluka Analytical, Germany, and butylated hydroxytoluene (BHT) was acquired from SAFC, Germany. Propylene glycol was purchased from Acros Organics, UK and vaseline white was acquired from Riedel-de Haën, Germany. Glycerol monostearate-self emulsifier (GMS-SE) and Carbopol ultrez 21 (acrylates/C10-30 alkyl acrylate crosspolymer, The Lubrizol Corporation, USA), were kindly received from Manufacturing unit, Pharmacy Department, Faculty of Medicine Siriraj Hospital, Thailand.

2.1.3 Membranes for permeation study

Fresh porcine abdominal skin was obtained from B&J Pigs Ltd, Somerset, UK. Excessive hair was carefully trimmed using scissors. After cleaning with running cold water, the skin was dermatomed (Zimmer electric dermatome, Oklahoma, USA) to a nominal thickness of 750 µm. The dermatomed skin was sealed in a plastic bag and stored at -20°C until use.

Artificial membranes, for the *in vitro* release test (IVRT), were a Tuffryn® membrane filter 145 µm thick with 0.45 µm pore size (HT-450, Pall Corporation, Mexico) and a Spectra/Por® Dialysis membrane (MWCO: 1000, wet in 0.05% sodium azide, nominal flat width 45 mm, 29 mm in diameter, volume/length: 6.4 mL·cm^{-1}, length: 10 m/33 ft, Spectrum® Laboratories, California, USA).

2.1.4 Human subjects

Six healthy volunteers aged between 25 and 31 years (4 females and 2 males), with no history of skin disease, no visible skin abnormalities and no prior skin treatment in the preceding 4 weeks, participated in the study. Participant information documents were provided to all volunteers prior to the investigations. Written informed consent was received from every volunteer before performing the experiments. The study protocol was approved by the Bath Local Research Ethics Committee, University of Bath (see Appendix 15).

The experiments were performed on both ventral forearms. The volunteers were instructed to avoid application of any creams, cosmetics, or washing products to either forearm during the course of the experiments.

2.2 Preparations

2.2.1 Preparation of standard solutions

Stock solutions (0.1 mg·mL^{-1}) of the phenolic standards were prepared by dissolution in methanol followed by sonication for 30 minutes where necessary (Fisherbrand® FB11002, Thermo Fisher Scientific Inc., UK). Each analyte stock solution was diluted with methanol to appropriate concentrations for the establishment of calibration curves and validation tests. All standard solutions were filtered through a 0.45 µm nylon membrane (Chronus® filter,

LabHut Ltd., UK) before HPLC-MS or HPLC-PDA analysis. Both stock and diluted solutions were stored at 4°C.

2.2.2 Preparation of plant sample solutions

The dried extracts of both *A. ebracteatus* and *C. petasites* were accurately weighed and separately dissolved in methanol at a concentration of 50 $mg \cdot mL^{-1}$ and sonicated for 30 minutes. After centrifugation at 4000 rpm for 20 minutes (U-32, Boeco, Germany), the supernatants of the individual samples were filtered through a 0.45 µm nylon membrane and diluted with methanol to appropriate concentrations prior to HPLC-MS or HPLC-PDA analysis. The filtered plant sample solutions were stored at 4°C.

2.2.3 Preparation of topical formulations

2.2.3.1 Solution

A solution containing the *C. petasites* extract at a concentration of 50 $mg \cdot mL^{-1}$ in 50% aqueous ethanol was prepared. The dried ethanolic extracts were weighed and dissolved in ethanol (50% of total solvent used in the formulation, v/v) by sonication for 60 minutes. After that, purified water was added to the solution to adjust the ethanol level to 50% (v/v) and sonicated for another 60 minutes and then centrifuged at 4000 rpm for 20 minutes. The supernatants were filtered through a 0.45 µm nylon membrane before use in the *in vitro* permeation tests. The solution was used within 24 hours of preparation.

2.2.3.2 Paste

A paste consisting of 50% *C. petasites*, 17% propylene glycol and 33% vaseline (w/w) was prepared. The dried ethanolic extracts were accurately weighed and pulverized with a mortar and pestle. Propylene glycol was added to wet the powders and then the wetted powders were thoroughly suspended into vaseline. The paste was used within 24 hours of preparation.

2.2.3.3 Cream and lotion

Excipients of the cream and lotion are listed in Table 2.1. The dried ethanolic extracts of *C. petasites* (CP) were accurately weighed and wetted with propylene glycol and glycerol in a glass mortar and pestle to form a paste (left until the emulsion base was prepared in the next step). The oil phase components, containing GMS-SE, cetyl alcohol, stearyl alcohol, mineral oil, Tween 60 and Span 60, were weighed into an appropriate beaker. The aqueous phase components, including Carbopol ultrez and water, were measured into a separate beaker. Both oil and aqueous phase components were heated until they were completely melted. The temperature of the oil phase was about 70°C (and never over 85°C), that of the aqueous phase was 75-80°C (5-10°C higher than the oil phase). The aqueous phase was then gradually poured into the oil phase and vigorously mixed with a stirring rod. When the emulsion was formed, gentle stirring was adopted to prevent bubbles. The paste of the plant extracts, prepared earlier, was then slowly added and incorporated into the emulsion. Concentrated paraben and BHT were subsequently added when the emulsion was congealed (about 40°C). The emulsion was cooled to room temperature before being stored in a light-protective and airtight container at 4°C.

An aqueous dispersion of Carbopol ultrez (2%, w/w) was prepared before formulating the emulsion. The powder was weighed and dispersed into hot water (two thirds of water content required). Cold water (one third of water content remaining) was added into the dispersion. Once a cloudy solution without lumps had formed, triethanolamine (TEA) was added dropwise into the solution until a gel was created, fully thickened, and transparent (pH = 7, using Whatman pH indicator papers, Fisher Scientific, UK). The polymer gel was stored at 4°C and used within 7 days.

Concentrated paraben was a preservative mixture of methyl paraben (10 g) and propyl paraben (2 g) in propylene glycol (100 mL). The stock solution was stored in a light protective and airtight container at 4°C.

Table 2.1 Excipients of cream and lotion formulations.

Excipient	Quantity (%, w/w)			
	10% CP cream	**Control cream**	**10% CP lotion**	**Control lotion**
C. petasites (dried ethanolic extracts)	10	-	10	-
GMS-SE	5	5	1	1
Cetyl alcohol	5	5	1.5	1.5
Stearyl alcohol	2.5	2.5	-	-
Mineral oil	15	15	15	15
Tween 60	4	4	4	4
Span 60	1	1	1	1
Carbopol ultrez (2% w/w stock gel)*	3	3	3	3
Propylene glycol	4	4	3	3
Glycerol	3	3	-	-
Concentrated paraben*	1% v/w	1% v/w	1% v/w	1% v/w
BHT	0.02	0.02	0.02	0.02
Purified water qs. to	100	100	100	100

* pre-preparation is required before formulating cream and lotion

2.2.3.4 Analysis of topical formulations

The cream and lotion (300 and 600 mg) were extracted by liquid-liquid partition with 10 mL acetonitrile and 30 mL hexane. The mixture was shaken vigorously and sonicated for 10 minutes before separating by a 50 mL glass funnel. The acetonitrile fraction was then collected and filtered through 0.45 µm nylon membrane prior to UV detection.

2.2.4 Preparation of Tris buffer and receptor solution for *in vitro* percutaneous tests

A stock solution of 50 mM Tris buffer consisted of Trizma® base (0.18 g) and Tris-HCl (1.35 g) dissolved in water (200 mL). The stock solution was diluted with water to obtain a 5mM Tris buffer (pH 7.4 measured by a pH meter, 420 series, Thermo Orion, Hugli-Labortec, Switzerland) for *in vitro* experiments. The buffer solutions were stored at 4°C and used within 14 days.

The receptor solution used for *in vitro* diffusion tests was a 1:4 v/v mixture of ethanol and 5 mM Tris buffer. The mixture was sonicated for 30 minutes before use.

2.2.5 Maximization of sample concentration by freeze drying

The samples from receptor solutions, obtained from *in vitro* percutaneous experiments, and the samples from tape extractions, obtained from *in vivo* tests, were frozen at approximately -70°C in a cooling bath (containing a liquid mixture of dry ice and acetone). The frozen samples were subsequently freeze dried below -40°C under vacuum (Modulyo freeze dryer, Thermo Fisher Scientific Inc., USA). The freeze dried samples were stored at 4°C.

The container for freeze drying was chosen by the sample size. 7.5 mL samples (as obtained from the receptor compartment of the skin permeation apparatus) were placed into a round-bottom flask (Round bottom flasks-quickfit QFH-162-B (FR100/4S) 100 mL-socket 29/32, Fisher Scientific, UK) and subsequently redissolved in 0.75 mL methanol. Samples with a volume between 1 to 1.5 mL (as obtained from extracting tape-strips) were placed into 2 mL amber eppendorf self-locked microcentrifuge tubes, and subsequently redissolved in 100 µL methanol. The samples were sonicated for 30 minutes prior to HPLC-MS or HPLC-PDA analysis. The remaining samples were freeze dried again and stored in the dark at 4°C.

2.3 Thin layer chromatography (TLC) and high performance thin layer chromatography (HPTLC)

2.3.1 Thin layer chromatography (TLC)

A TLC method was used to screen compounds from numerous sample fractions. 5 µL of individual sample solutions in ethanol were spotted on a TLC plate (aluminium-backed sheet coated with silica gel $60F_{254}$, 20x20 cm, Merck, Germany). The mobile phase comprised ethyl acetate, methanol, formic acid in a ratio of 50:3:3 v/v/v. The developed plate was visualized under ultraviolet (UV) light at 254 and 366 nm and then sprayed with ethanolic ferric (III) chloride reagent before being observed under visible light.

2.3.2 High performance thin layer chromatography (HPTLC)

A HPTLC system comprising a Camag horizontal developing chamber (CAMAG Scientific, Switzerland) and HPTLC plate (HPTLC-Platten, 10x10 cm, Keiselgel 60F_{254}, Merck, Germany) was used. The mobile phase was 50:4:4:2.5 v/v/v/v ethyl acetate, methanol, water and formic acid. 5 µL of individual standard solution (1 mg·mL^{-1}) and 5 and 10 µL of each plant solution in methanol (50 mg·mL^{-1}) were loaded on to the plate. The bands were visualized under UV light at wavelengths of 254 and 366 nm by a Camag Reprostar 3-110709, and then sprayed with ethanolic ferric (III) chloride to capture the bands under visible light. Thereafter, the bands were sprayed again with sulphuric acid (H_2SO_4) solution in ethanol (10%, v/v) and detected at 366 nm.

2.4 High performance liquid chromatography-mass spectrometry (HPLC-MS) and high performance liquid chromatography-photodiode array detection (HPLC-PDA)

2.4.1 HPLC-MS

2.4.1.1 Analytical chemistry

Experiments were mainly performed on a Shimadzu HPLC-2010A HT system (Shimadzu Corp., Kyoto, Japan) consisting of an autosampler, vacuum degasser, and UV detector which was set at the detection wavelengths of 260 and 330 nm (resulting from HPLC-PDA results of individual standards).

The HPLC was connected to a Shimadzu MS-2010EV system (Shimadzu Corp., Kyoto, Japan) with a dual source of electrospray ionization and atmospheric pressure chemical ionization (ESI/APCI, DUIS-2010, Japan). Ionization was achieved in both negative- and positive-ion-modes with detector voltage set at 1.5 kV. Nitrogen was used as a nebulising gas, heated to 480°C and delivered at a flow rate of 1.5 L·mL^{-1}. MS signals were collected in the scan ion-monitoring mode between 50-1000 m/z for identification of chemical components and the single ion-monitoring (SIM) mode was used for quantification of individual compounds.

The column used was a Dionex Acclaim® 120 (C18, 5 µm, 150 x 4.6 mm i.d.). A combination of acetonitrile (A) and 0.1% aqueous acetic acid (v/v, B) was used as a mobile phase with an optimized gradient system. The injection volume was 20 µL and the flow rate was 0.5 mL·min^{-1}. The column temperature was maintained at 35°C

throughout the analysis. All data acquired were processed by the LabSolutions LCMS Software (Shimadzu Corp., Kyoto, Japan).

2.4.1.2 Optimization of analytical method

The HPLC-MS methods displayed in Table 2.2 were developed to identify and quantify phenolic compounds. Indicators for system evaluation were resolution of peaks, peak height, peak area, and peak shape from chromatograms detected by UV and MS detectors. Standard solutions (1 $\mu g \cdot mL^{-1}$) of caffeic acid, vanillic acid, verbascoside and chrysin, and individual sample solutions (0.1 $mg \cdot mL^{-1}$) of *A. ebracteatus* and *C. petasites* in methanol were used to develop the HPLC-MS conditions. The gradient system no. 9 was chosen and established as the optimized HPLC-MS method for the study.

Table 2.2 The mobile phases tested in selection of solution systems.

Condition no.	Solution system	Mobile phase	Programme
1	Isocratic	Acetronitrile (A) and 0.1% aqueous acetic acid (B)	20% A for 30 min
2	Isocratic	Acetronitrile (A) and 0.1% aqueous acetic acid (B)	25% A for 30 min
3	Isocratic	Acetronitrile (A) and water (B)	25% A for 30 min
4	Isocratic	Methanol (A) and 0.1% aqueous acetic acid (B)	25% A for 30 min
5	Gradient	Formic acid/acetronitrile at pH 3 (A)	5% A for 20 min
		Formic acid/water at pH 3 (B)	5-40% A for 30 min
			40-95% A for 5 min
			95% A for 5 min
6	Gradient	Acetronitrile (A) and 0.01% aqueous acetic acid (B)	70-100% A for 2 min
			100% A for 20 min
			100-70% A for 1 min
			70% A for 4 min
7	Gradient	Acetronitrile (A) and 0.1% aqueous acetic acid (B)	20% A for 20 min
			20-95% A for 15 min
			95% A for 10 min
			20% A for 15 min
8	Gradient	Acetronitrile (A) and 0.1% aqueous acetic acid (B)	20-80% A for 15 min
			95% A for 10 min
			100% A for 20 min
			95-20% A for 15 min
9	Gradient	Acetronitrile (A) and 0.1% aqueous acetic acid (B)	20% A for 9 min
			20-60% A for 6 min
			60% A for 5 min
			60-95% A for 10 min
			95% A for 5 min
			20% A for 25 min
10	Gradient	Acetronitrile (A) and 0.1% aqueous acetic acid (B)	15% A for 12 min
			15-55% A for 5 min
			55% A for 8 min
			55-95% A for 10 min
			95% A for 5 min
			15% A for 20 min
11	Gradient	Acetronitrile (A) and 0.1% aqueous acetic acid (B)	25% A for 9 min
			25-55% A for 5 min
			55% A for 8 min
			55-95% A for 10 min
			95% A for 5 min
			25% A for 20 min

2.4.2 HPLC-PDA

2.4.2.1 Analytical chemistry

The HPLC-PDA system comprised an ASI-100 automated sample injector, thermostatted column compartment TCC-100 and PDA-100 photodiode array detector (Dionex® Ltd., UK). The UV detection wavelengths were set at 260 and 330 nm for quantification and the maximum wavelengths (λ_{max}) of each peak were detected by a wavelength scan from 240 to 360 nm for peak confirmation.

A HiQ Sil C18 HS column (C18, 5 µm, 150 x 4.6 mm i.d., Kyatech, Japan) was used and the temperature was maintained at 35°C. A mobile phase consisting of acetronitrile (A) and a mixture of 0.1% aqueous acetic acid and acetonitrile (v/v, 80:20, B) was used in an optimized gradient system with a flow rate of 0.5 mL·min^{-1}. 20 µL of each sample was injected. Chromatograms were interpreted with Chromeleon software (Dionex® Ltd., UK).

2.4.2.2 Optimization of analytical method

The HPLC-PDA conditions were slightly changed from those which had been optimized for HPLC-MS. Acetonitrile (A) and a mixture of 0.1% aqueous acetic acid and acetronitrile (v/v, 80:20, B) were combined as the mobile phase in a gradient system of 0% A for 9 min, 0-50% A for 6 min, 50% A for 5 min, 50-94% A for 10 min, 94% A for 5 min and 0% A for 25 min. Retention times (t_R) and UV peak detection using HPLC-PDA were compared with those using HPLC-MS. Standard solutions (1 µg·mL^{-1}) of caffeic acid, vanillic acid, verbascoside and chrysin in methanol were used for preliminary method assessment.

2.5 Nuclear magnetic resonance spectroscopy (NMR)

All ethanolic extracts, solvent partition fractions, and phenolic standards were dissolved in appropriate solvents (e.g., deuterated-methanol (CD_3OD), deuterated-chloroform ($CDCl_3$) and deuterium oxide (D_2O)). ^{1}H NMR (500 MHz) data was obtained on a Varian Mercury spectrometer. Chemical shifts (δ) were recorded in parts per million (ppm).

2.6 *In vitro* skin penetration experiments

The skin permeation of compounds in the plant extracts was determined using vertical, glass Franz diffusion cells (PermeGear, Inc., Bethlehem, PA, USA). The exposed membrane surface area was 1.77 cm^2 and the receptor volume was 7.5 mL. The receptor solution was a mixture of ethanol and 5 mM Tris buffer in ratio of 1:4 v/v, at pH 7.3 (slightly less than 7.4 due to the presence of ethanol). Frozen dermatomed pig abdominal skin was thawed for 30 minutes before use and examined visually for punctures or defects. The use of skin stored frozen has been validated in earlier work (Hawkins and Reifenrath, 1986). The skin was stripped with one adhesive tape (3.5 cm x 3.5 cm, Scotch book tape, 3M, MN, USA) to remove SC disjunctum before being mounted into the Franz cell. The stratum corneum (SC) side, the outermost surface, faced the donor compartment. After temperature equilibration at 37°C, the formulations were applied to the skin surface and occluded with Parafilm™ (Bemis®, USA). The amount of drug applied, sampling volume and sampling time of each experiment are in Table 2.3. At each sampling time, the whole receptor solution volume was removed and replaced with fresh buffer. The samples were stored at 4°C under light protection before quantitatvie analysis. At the end of the experiment, the skin was removed from the Franz cell and the remaining formulation was wiped off with an isopropyl alcohol swab (70% isopropyl alcohol; Sterets®, Medlock Medical Ltd., UK). The skin was then pinned to a polystyrene board and left to air dry for 3 hours. A template with a circular aperture (1.4 cm diameter, Scotch book tape, 3M, MN, USA) was positioned over the treated area before stripping the SC using tapes. Six replicates were performed with each formulation.

Table 2.3 Amounts of drug applied, sampling volumes and times of *in vitro* permeation tests for each topical formulation.

Formulation	Amount of drug applied (g)	Sampling volume (mL)	Sampling time (h)
50 $mg \cdot mL^{-1}$ CP solution	1 mL	7.5	3, 6, 24
50% CP paste	0.2 g	7.5	3, 6, 24
No formulation applied (skin control)	-	7.5	3, 6, 24
10% CP cream, 10% CP lotion, control cream, control lotion	0.2 g	7.5	6

2.7 *In vivo* skin penetration experiments

The passive diffusion of constituents of the plant extracts into the SC was studied on healthy volunteers. The study was conducted over 2 days and examined 2 different formulations. On Day 1, one arm was used for the first formulation, while the second formulation was applied to the other arm on Day 2 (Table 2.4).

Table 2.4 Experiment design over 2 days.

	Morning (apply drug)				**LUNCH**	**Afternoon (remove drug and tape strip)**			
	Site 1	**Site 2**	**Site 3**	**Site 4**		**Site 1**	**Site 2**	**Site 3**	**Site 4**
Day 1, Forearm 1	9.00	9.45	10.30	11.15		15.00	15.45	16.30	17.45
Day 2, Forearm 2	9.00	9.45	10.30	11.15		15.00	15.45	16.30	17.45

The ventral forearms were cleaned with an alcohol wipe and the volunteer was acclimatized to the treatment venue for 30 minutes. Four skin sites (3 treated + 1 blank, control) were delineated on each arm at least 4 cm above the wrist and a minimum of 4 cm below the elbow. Each treatment site (6 cm^2 in area, Fig. 2.1) was demarcated with a rectangular frame cut from a self-adhesive foam (1.57 mm in thickness; 3M, USA) and applied to the ventral forearm with the long dimension oriented across the forearm. One tape strip was discarded before drug application to remove SC disjunctum. Good contact between tape and skin was ensured by running a weighted roller (6 cm wide, 140 g cm^{-2}) over the tape several times before its removal. A fingertip cut from a laboratory glove (nitrile and powder-free glove; Kimtech, UK) was used to distribute the cream/lotion over the demarcated area. The sites were treated at 45-minute intervals. Approximately 0.2 g of the product (containing 10% w/w *C. petasites* extracts) was applied to sites 1-3, while the blank (cream/lotion base without *C. petasites*) was applied to site 4. The actual amount applied was determined by weighing the fingertip before and after drug application. All sites were occluded by covering the foam frame with Parafilm™ and securing it to the skin with a self-adhesive fabric dressing (Mefix®, SCA Molnlycke Ltd., Sweden). The drug application time was 6 hours.

At the end of the experiment, the dressing, Parafilm™ and foam frame were removed. Excess cream/lotion was gently wiped away with tissue (Fort James Ltd., UK), and then swabbed three times with alcohol wipes. The skin was finally left to air dry for 1 minute

before a new thin foam frame (dimension of the cut out inside are 1.5 cm x 2 cm, 0.75 mm in thickness; 3M, USA) was placed over each cleaned site in the same position as the original frame (Fig. 2.1). Subsequently, the tape stripping procedures were continued.

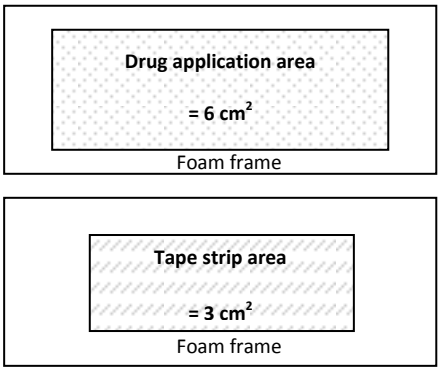


Fig. 2.1 Illustration of drug application and tape strip area (adapted from N'Dri-Stempfer *et al.* (2009)).

2.8 Tape stripping and tape extraction

Tape stripping was performed at the end of the *in vitro* and *in vivo* percutaneous experiments. The stripped area was designated by the template depending on the individual test as described above. First, transepidermal water loss (TEWL) was measured (AquaFlux® evaporimeter, Biox System Ltd., UK) to obtain an initial value. An adhesive tape strip (2.5 cm x 2.5 cm; Permacel J-LAR®) was applied to the skin and pressed firmly down using the weighted roller. The tape was removed quickly from the skin and the TEWL value was then measured again. The procedure was repeated until TEWL reached 4 times the initial value (when approximately 75% SC had been removed (Kalia *et al.*, 2000)) or when 30 strips had been taken.

The mass of skin removed on each tape was determined by weighing the tapes on a microbalance (Satorius model SE2-F, Sartorius AG, Germany), before and after application to the skin. Before weighing, the tapes were stored at room temperature for at least 12 hours and static electricity was removed (R50 discharging bar and ES50 power supply, Eltex Elektrostatik GmbH, Weil am Rhein, Germany).

After weighing, the tapes were grouped for methanol extraction. The first and second tapes were individually analysed, while the remaining tape strips were combined into groups of 2-4 tapes depending on the total number collected in each experiment. Groups containing

1-2 tapes were extracted with 1 mL of methanol, those with 3-4 tapes were extracted into 1.5 mL. Extraction involved shaking overnight (IKA HS 260 Basic shaker, IKA® Werke GmbH & Co., KG, Germany). The extracted solutions were then filtered through a 0.45 µm nylon membrane, and either directly injected, or concentrated by freeze drying before injection into an HPLC-MS or HPLC-PDA. If not processed at once, the samples were freeze dried and stored at 4°C before analysis.

2.9 *In vitro* release test (IVRT) on cream and lotion formulations

Vertical, glass Franz diffusion cells, with a surface area of 1.77 cm^2, and a receptor volume of 7.5 mL, were used. A synthetic membrane (Tuffryn® membrane filter 145 µm thick with 0.45 µm pore size, HT-450, Pall Corporation, Mexico) was soaked in the receptor solution for 30 minutes before being mounted into the cells. The receptor solution was a 1:4 v/v mixture of ethanol and 5mM Tris buffer at pH 7.4, and maintained at 37°C. Approximately 1 g of cream or lotion containing 10% w/w *C. petasites* was applied to the membrane surface facing the donor compartment. 300 µL of receptor solution was subsequently withdrawn at 15 min, 30 min, 45 min, 1 h, 1.5 h, 2 h, 3 h, 4 h, 5 h, 6 h, 8 h, 24 h, 32 h, 48 h, 56 h, and 72 h. The samples were filtered through a 0.45 µm nylon membrane and freeze dried. Before analysis by HPLC-MS or HPLC-PDA, the freeze dried samples were re-dissolved in an appropriate amount of methanol. Six replicates were performed.

2.10 Mathematical methods

2.10.1 Prediction of maximum flux (J_{max})

A maximum possible flux (J_{max}, $\mu g \cdot cm^{-2} \cdot h^{-1}$) of chemical transport was calculated from an algorithm derived from Fick's first law of diffusion as follows:

$$J_{max} = k_p \cdot C_{sat,W} \qquad \text{Eq. 2.1}$$

where k_p is the compound's permeability coefficient ($cm \cdot h^{-1}$) and $C_{sat,W}$ is the saturation solubility of the compound in water ($\mu g \cdot cm^{-3}$). The k_p value is estimated by the Potts and Guy equation (Eq. 2.2) (Potts and Guy, 1992).

$$\log k_p = -2.72 + 0.71 \cdot \log P - 0.0061 \cdot MW \qquad \text{Eq. 2.2}$$

where P is the compound's octanol-water partition coefficient (no units) and MW is its molecular weight (Da).

However, because the viable epidermis can represent a significant barrier to the penetration of lipophilic compounds, the Potts and Guy estimated k_p (which assumes the transport across the skin is controlled uniquely by the SC) is corrected as proposed by Cleek and Bunge (1993) as follows:

$$k_p^{corr} = \frac{k_p}{1 + \frac{k_p \cdot \sqrt{MW}}{2.6}} \qquad \text{Eq. 2.3}$$

It follows that J_{max} for the putative active species in the plant extracts can be predicted from Eqs. 2.1, 2.2 and 2.3 using readily available or calculable values of MW, log P and $C_{sat,W}$ (ALOGPS 2.1 algorithm, 2001; Chemspider).

2.10.2 Estimation of total SC thickness

The thickness of each layer of SC removed was deduced from Eq. 2.4, where the SC density is assumed to be 1 g·cm^{-3} (Anderson and Cassidy, 1973).

$$\text{SC layer thickness removed} = \frac{\text{mass of SC removed on tape}}{\text{area of SC stripped} \cdot \text{SC density}} \qquad \text{Eq. 2.4}$$

Total SC thickness was determined from the change in TEWL as a function of the amount of SC removed, i.e.,

$$TEWL_x = \frac{K \cdot D \cdot \Delta C}{(L - x)} \qquad \text{Eq. 2.5}$$

and, hence,

$$\frac{1}{TEWL_x} = \frac{L}{K \cdot D \cdot \Delta C} - \left[\frac{1}{K \cdot D \cdot \Delta C}\right] x \qquad \text{Eq. 2.6}$$

$TEWL_x$ is the measured transepidermal water loss (g·m^{-2}·h^{-1}) when x µm of SC has been stripped away; K is the SC-viable epidermis partition coefficient of water (no units); D is the diffusivity of water in the SC (cm^2·h^{-1}); ΔC is the concentration gradient of water across the SC (~ 1 g·cm^{-3} (Kalia *et al.*, 2000)); L is the total SC thickness (µm).

A representative set of data showing the change in $1/TEWL_x$ as a function of the cumulative SC thickness removed (x) is in Fig. 2.2. The total SC thickness (L) is obtained from the x-intercept.

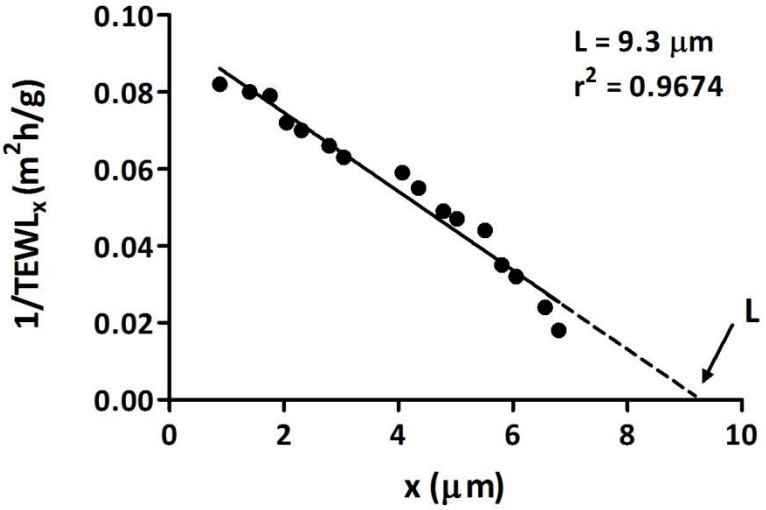


Fig. 2.2 Example graph of $1/TEWL_x$ as a function of the cumulative SC thickness removed (x) using a linear fit model.

An alternative data analytical approach, by which to determine L from the change in TEWL as the SC is progressively stripped, involves direct fitting of the results to a so-called baseline-corrected model (Russell *et al.*, 2008)

$$\mathrm{TEWL_x} = \mathrm{B} + \frac{\mathrm{K} \cdot \mathrm{D} \cdot \Delta\mathrm{C}}{(\mathrm{L} - \mathrm{x})} \qquad \text{Eq. 2.7}$$

where B is a constant that reflects the fact that increases in TEWL are often not measurable until the stratum disjunctum has been removed. An example set of data fitted by Eq. 2.7 is shown in Fig. 2.3.

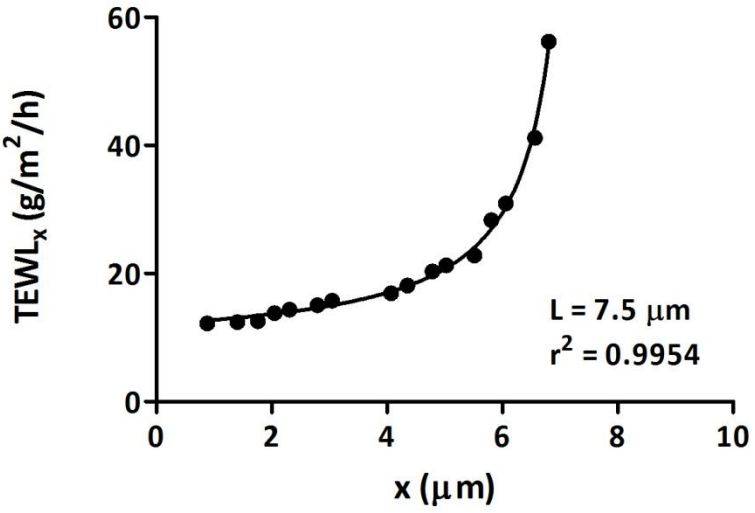


Fig. 2.3 Example graph of $TEWL_x$ as a function of the cumulative SC thickness removed (x) using a baseline-corrected non-linear fit model (re-plotted data from Fig. 2.2).

2.10.3 Drug release determination

Release profiles were analysed using the classic Higuchi equation (Eq. 2.8) (Higuchi, 1961), which describes the cumulative amount compound released per unit area (M, μg· cm^{-2}) as a function of the square root of time ($t^{1/2}$, $h^{1/2}$).

$$M = [2C_t D C_d]^{1/2} t^{1/2} \qquad \text{Eq. 2.8}$$

where D is the diffusion coefficient of the compound in the formulation ($cm^2 \cdot s^{-1}$); C_t is the total concentration of dissolved and undissolved compound in the formulation ($\mu g \cdot cm^{-3}$); C_d is the solubility of the compound in the homogenous formulation ($\mu g \cdot cm^{-3}$).

2.11 Validation and statistical analysis

2.11.1 Limits of detection and quantification (LOD and LOQ)

Each standard solution was diluted and measured in triplicate to assess a signal-to-noise ratio (S/N). The S/N was the ratio of the height of the chromatographic signal above the baseline and the height of the baseline noise measured more than 30 seconds before and after the peak to avoid any peak tails. The concentration with S/N ≥ 3 was defined as LOD and that with S/N ≥ 10 was identified as LOQ.

2.11.2 Calibration curves

Separate calibrations were carried out for HPLC-MS and HPLC-PDA assays. At least six concentrations and three independent preparations of phenolic standards in the range of 0.25 – 20 ng were injected into the HPLC-MS detector. 0.25 ng – 2 µg of standard mixtures were subjected to HPLC-PDA detection. Calibration curves were obtained by plotting the areas under the curves (AUCs) against concentration and the equation of the line determined by linear regression (detail in Appendices 2 and 3). The curves were used only within the linear range.

2.11.3 Precision

Three different concentrations (low, middle, and high examples on the calibration curves) of individual phenolic standards were measured five times a day to determine intra-day variability. The standards were also analysed twice a day on three consecutive days in order to obtain inter-day variability. The results were expressed in terms of relative standard deviation (RSD).

2.11.4 Statistical analysis

All statistical analyses were performed using GraphPad Prism® version 5 (GraphPad Software Inc., CA, USA). Calibration curves were analysed with linear regression. Datasets were expressed as mean ± SD (standard deviation) and compared for statistical significance at $P \leq 0.05$ with two-way ANOVA and Bonferroni post-tests.

Chapter 3 Identification and quantification of polyphenols in *A. ebracteatus* and *C. petasites*

3.1 Study purpose

Polyphenolic compounds are abundant in plants with key functions in growth and development. They absorb ultra violet (UV) light efficiently due to their extensive chromophores. Flavonoids are one type of phenolic compounds with biological activity. They are well-known as strong antioxidants with free radical scavenging and metal chelating activities (Perron and Brumaghim, 2009; Robak and Gryglewski, 1996; Wuguo *et al.*, 1997), and are extensively used in dermatological and cosmetological applications (Arct *et al.*, 2002; Arct and Pytkowska, 2008; Bonina *et al.*, 1996; Cimino and Saija, 2005; Lin *et al.*, 2008).

As there are a number of constituents in an herbal product which might contribute to its therapeutic effects, it is vital to determine as many of the substances present as possible, to study their bioactivity, and to ensure quality control. Several analytical techniques have been applied including high performance thin layer chromatography (HPTLC), high performance liquid chromatography (HPLC) with both photodiode array (PDA) detection and mass spectrometric (MS) detection, and nuclear magnetic resonance (NMR) spectroscopy. The chromatographic or spectroscopic profile may be used to distinguish the chemically distinct compounds present in various samples, especially trace levels, and allow their successful authentication and identification.

The study aims are:

- To establish HPTLC and NMR fingerprints for batch quality control.
- To identify and quantify polyphenolic compounds in the plants with HPLC-MS, HPLC-PDA and NMR.

3.2 Results and discussion

3.2.1 Establishment of herbal HPTLC pattern for batch quality control

HPTLC patterns for each plant were used as an easy method of batch quality control. The use of several mobile phases and visualization methods generated a preliminary idea of the chemical constituents in plant samples. The mobile phase compositions were based on polarities of the standards used in this experiment.

HPTLC fingerprints in developing solvent (50:4:4:2.5 v/v/v/v ethyl acetate: methanol: water: formic acid), visualized under: (a) UV 254 nm, (b) UV 366 nm, (c) visible light after spraying with ethanolic ferric (III) chloride ($FeCl_3$), (d) visible light after spraying with $FeCl_3$ and sulphuric acid (H_2SO_4), and (e) UV 366 nm after spraying with $FeCl_3$ and H_2SO_4, revealed the characteristic bands of *A. ebracteatus* (AE) and *C. petasites* (CP). $FeCl_3$ solution was sprayed to identify phenol, flavonoids, tannins and plant acids (Reich and Schibli, 2007). Fe^{3+} forms a coordination complex with the hydroxyl groups of polyphenols (Fig. 3.1) resulting in a range of colours under visible light (deep violet to orange). Flavonoids particularly gave a yellow colour. Terpenoids were indicated and distinguished from flavonoids after being sprayed with H_2SO_4 reagent and heating the HPTLC plates (Scott, 2000-2009). Coloured bands of terpenoids appeared due to an increase of conjugated double bonds.

The plant extracts and phenolic standards were spotted on the HPTLC plates as shown in Table 3.1.

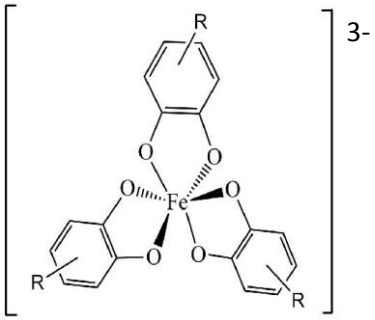


Fig. 3.1 Example of a coordination complex between Fe^{3+} and a cathecol-type flavonoid.

Fig. 3.2-A shows the similarities at two different concentrations (0.25, and 0.5 mg) and the repeatability of 0.5 mg plant extracts is presented in Fig. 3.2-B. The fingerprints reflect the reliability of this screening method for different batches. They also show the presence of

phenolic compounds in both plants extracts from a comparison of the colours and positions of sample bands with those of reference markers. The suspected phenolic constituents in the CP were verbascoside, hispidulin, luteolin, nepetin (Fig. 3.2-B), apigenin (Fig. 3.3-A), diosmetin, chrysoeriol and vanillic acid (Fig. 3.3-B). The hispidulin band of CP extract in Fig. 3.2-B-e had a dark colour in the middle surrounded by fluorescence showing a quenching effect. This effect normally resulted from overloading of samples and it can thus be assumed that the CP extract contains a high concentration of hispidulin compared to the other phenolic compounds in the plant extract. The AE extract may contain verbascoside (Fig. 3.2-B) and vanillic acid (Fig. 3.3-B).

Table 3.1 HPTLC track labelling.

Fig.	Panel	Lane 1	Lane 2	Lane 3	Lane 4	Lane 5	Lane 6	Lane 7
3.2	A	4-Coumaric acid	Gallic acid	AE (0.25 mg[a])	AE (0.5 mg[b])	CP (0.25 mg[a])	CP (0.5 mg[b])	5,7-Dimethoxy coumarin
	B	Verbascoside	Hispidulin	AE (0.5 mg[b])	CP (0.5 mg[b])	Luteolin	Scutellarein	Nepetin
3.3	A	Apigenin	Hesperetin	AE (0.5 mg[b])	CP (0.5 mg[b])	Cinnamic acid	Rosemarinic acid	Arbutin
	B	Cirsimaritin	Diosmesin	AE (0.5 mg[b])	CP (0.5 mg[b])	Chrysoeriol	Kaempferol	Vanillic acid
3.4	A	Rutin, 6-hydroxy flavone, quercetin	AE (batch 1, 0.5 mg[b])	AE (batch 2, 0.5 mg[b])	AE (batch 3, 0.5 mg[b])	AE (batch 4, 0.5 mg[b])	AE (batch 5, 0.5 mg[b])	Caffeic acid, Rosemarinic acid, ferulic acid
	B	Rutin, 6-hydroxy flavone, quercetin	CP (batch 1, 0.5 mg[b])	CP (batch 2, 0.5 mg[b])	CP (batch 3, 0.5 mg[b])	CP (batch 4, 0.5 mg[b])	CP (batch 5, 0.5 mg[b])	Caffeic acid, Rosemarinic acid, ferulic acid
3.5	A	Rutin, 6-hydroxy flavone, quercetin	AE (0.5 mg[b])	AE (petroleum fraction)	AE (ethyl acetate fraction)	AE (butanol fraction)	AE (water fraction)	Precipitate (insoluble in methanol)
	B	Rutin, 6-hydroxy flavone, quercetin	CP (0.5 mg[b])	CP (petroleum fraction)	CP (ethyl acetate fraction)	CP (butanol fraction)	CP (water fraction)	Precipitate (insoluble in methanol)

a = 50 mg·mL^{-1}, 5 µL; b = 50 mg·mL^{-1}, 10 µL

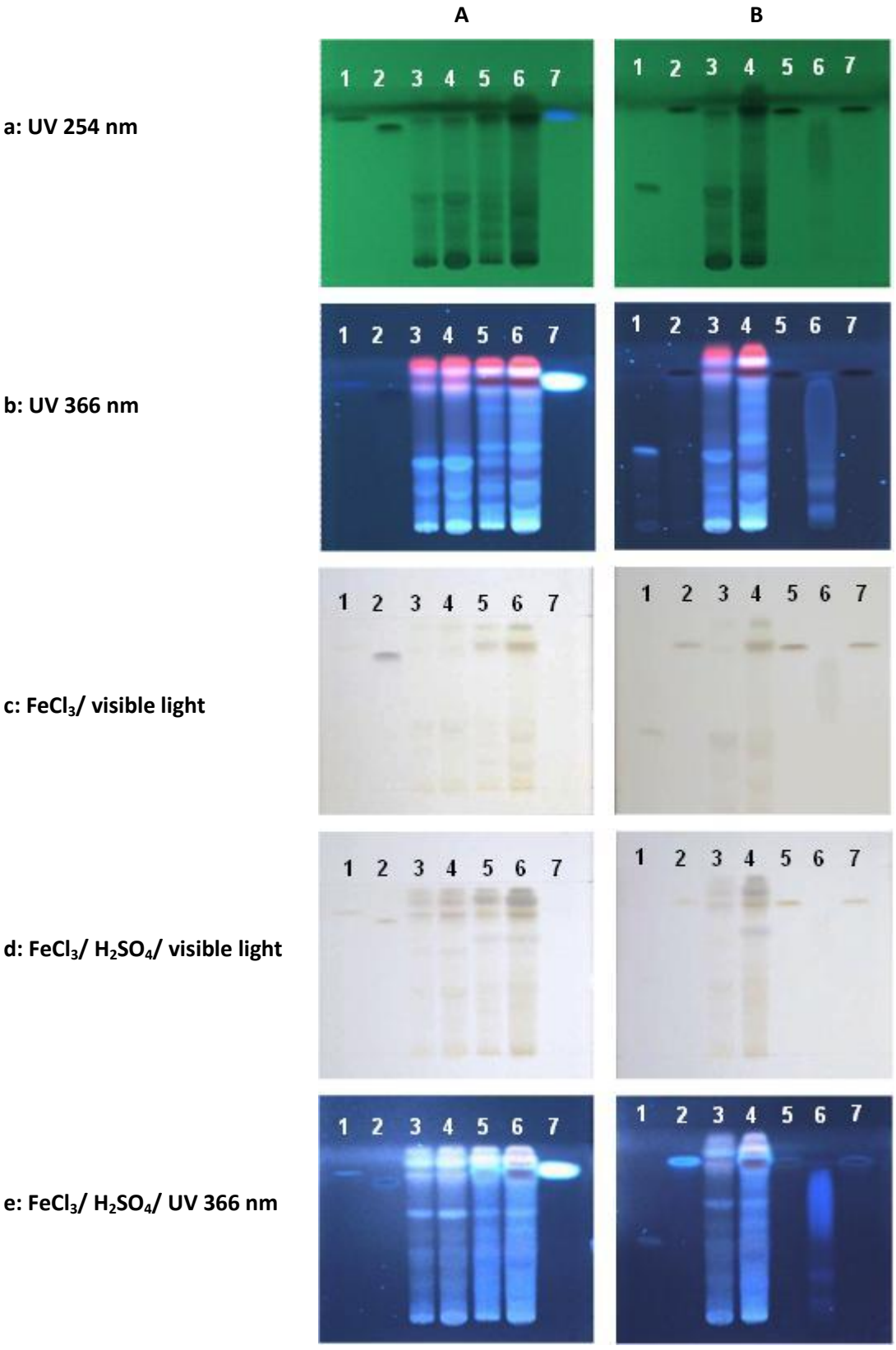


Fig. 3.2 HPTLC fingerprints of AE and CP. Track labelling is given in Table 3.1.

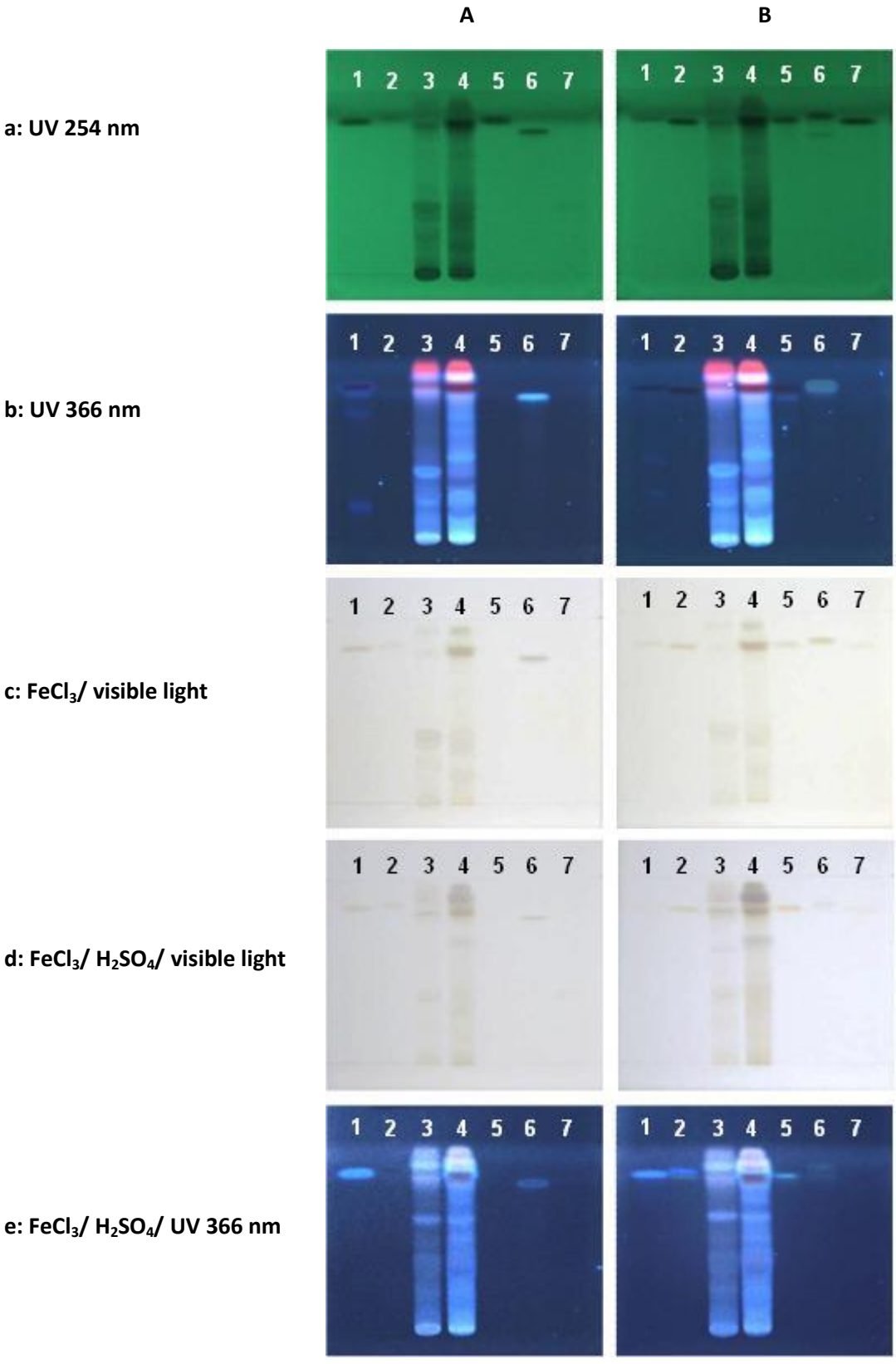


Fig. 3.3 HPTLC fingerprints of AE and CP. Track labelling is given in Table 3.1.

Five batches of the ethanolic extracts of crude powdered herbs were received from the Center of Applied Thai Traditional Medicine, Thailand and their yields are illustrated in Table 3.2. They contained substantial proportions of sugars and oils which could cause interferance in HPLC-MS and HPLC-PDA chromatograms and NMR spectra. Fractionation by liquid-liquid partition can separate chemical constituents of interest according in their polarities. Four fractions comprising petroleum ether, ethyl acetate, butanol and aqueous fractions were obtained and their yields are reported in Table 3.3.

Table 3.2 Yields of *A. ebracteatus* and *C. petasites* in five batches of ethanolic extracts.

Plant sample	**Weight of crude plant (kg)**	**% yield of each batch**					
		Batch 1	**Batch 2**	**Batch 3**	**Batch 4**	**Batch 5**	***Average ± SD***
A. ebracteatus	1.0	10.5	11.7	11.7	12.5	12.5	***11.8 ± 0.8***
C. petasites	1.0	4.0	2.7	3.1	2.9	3.1	***3.2 ± 0.5***

Table 3.3 Yields of *A. ebracteatus* and *C. petasites* in four fractions separated by liquid-liquid partition.

Plant sample	**Weight of ethanolic extract (g)**	**% yield of each fraction**			
		Petroleum ether	**Ethyl acetate**	**Butanol**	**Water**
A. ebracteatus	15.4	2.6	4.7	15.1	42.4
C. petasites	15.5	4.5	13.9	27.3	31.8

Quality controls of five different batches of AE and CP are shown in

Fig. 3.4. The similarity of different batches showed good reproducibility of the ethanolic extraction method. It is also clear from Table 3.3 that AE contained more polar substances (e.g., glycosides) than CP, whereas CP was likely to have a higher proportion of non-polar constituents (e.g., aglycones). Moreover, suspected bands of terpenoids were found in CP after being sprayed with H_2SO_4.

With respect to liquid-liquid partition, Fig. 3.5 shows that most of the non-polar compounds were detected in the ethyl acetate fraction. Likewise, most polar molecules were separated into the water fraction. The ethyl acetate fraction of AE showed possible phenolic aglycones or flavonoid monosaccharides, whereas, the butanol fraction tended to have more highly glycosylated phenolic compounds. On the other hand, both ethyl acetate and butanol fractions of CP showed similar patterns; only some phenolic glycosides were

better separated in the butanol fraction. Efficiency of the liquid-liquid partitioning method was insured from the tests of precipitates with no compounds of interest detected but may contain polysaccharides and/or inorganic salts.

All characteristic compounds that were tentatively identified from HPTLC screening were chosen for further evaluation and confirmation by HPLC-MS and HPLC-PDA.

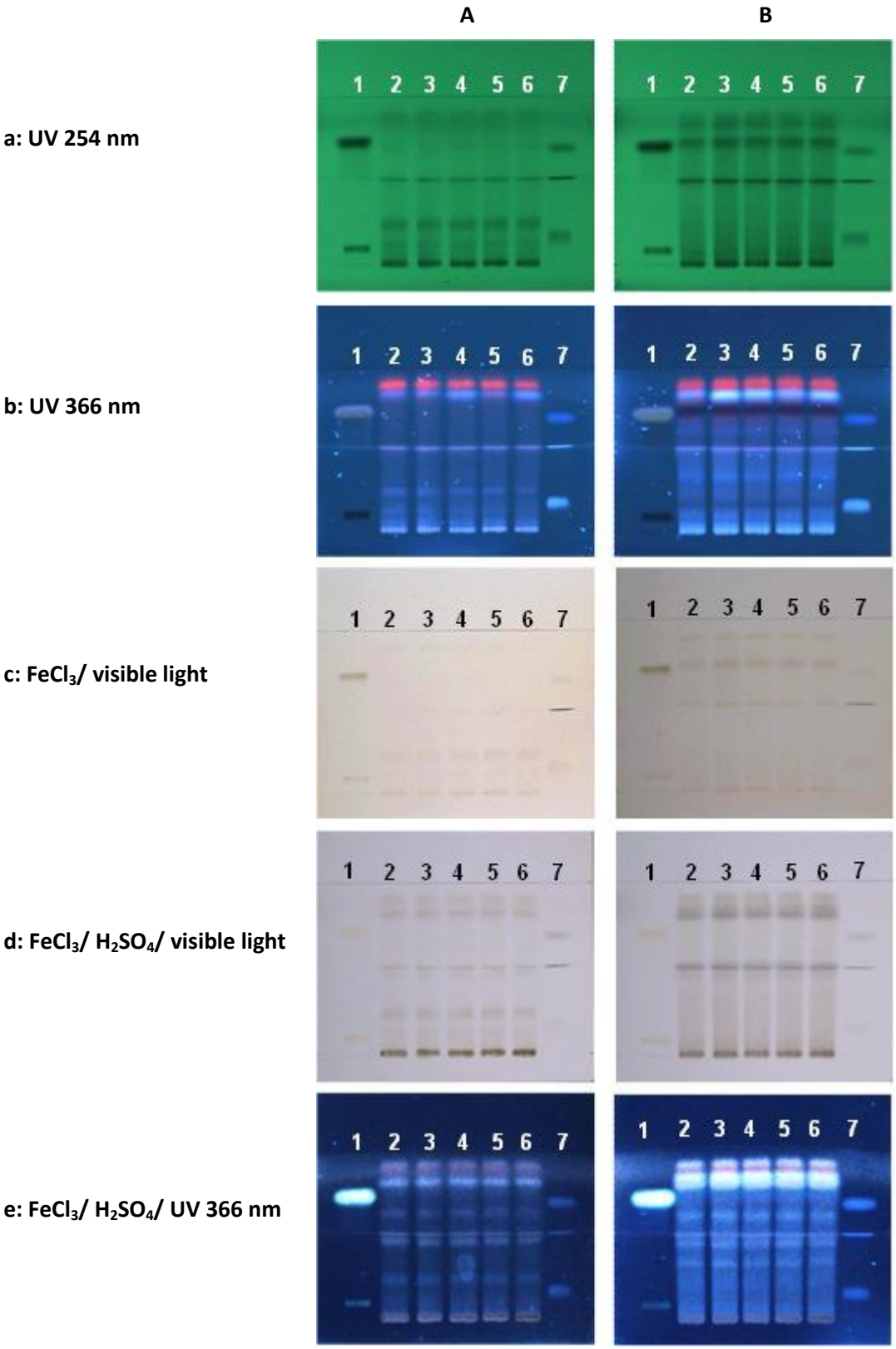


Fig. 3.4 Separations of five batches of the plants. Track labelling is given in Table 3.1.

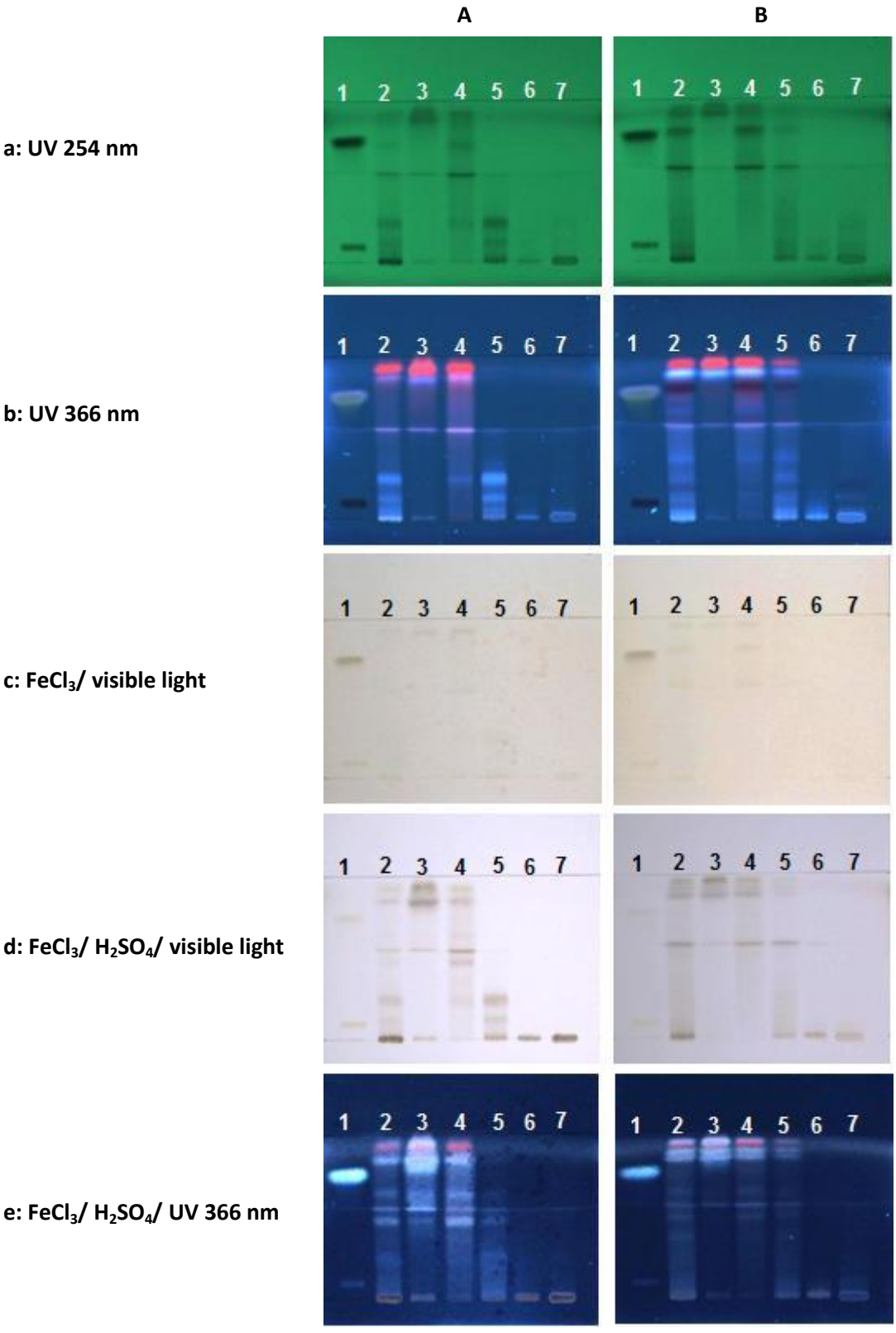


Fig. 3.5 Separations of liquid-liquid plant fractions. Track labelling is given in Table 3.1.

3.2.2 Screening natural compounds in the plant extracts by NMR spectroscopy

In addition to HPTLC fingerprints, the five batches of ethanolic extracts were also screened by NMR to characterise natural compounds and check their reproducibility. NMR spectra of all batches were the same (see Appendices 6 and 7); however, the ethanolic extracts contained very high amounts of sugars (spectral features in the region of 3-4 ppm) and oils (spectral features in the region of 0.5-2.5 ppm).

After a removal of sugars and oils by liquid-liquid partition, the four fractional extracts; petroleum ether, ethyl acetate, butanol and water fractions, were examined by NMR to broadly identify the components of each fraction (see Appendices 8 and 9). As expected, the presence of considerable amounts of sugars and oils was apparent in the water and petroleum ether fractions, respectively. The butanol fractions showed small peaks in the region of 6-8 ppm and other peaks in the sugar region at 3-4 ppm. This suggests the presence of phenols and their glycosides which may include some of the standards used in this study or other unknown phenolic compounds. The ethyl acetate fractions exhibited signals in the region of 5-6 ppm typical of alkene protons which may indirectly indicate the presence of terpenes, phytosteroids or unsaturated fatty acids. As a result, the NMR spectra supported the main focus on phenolic compounds in this study.

3.2.3 HPLC-MS chromatograms and validations of phenolic standards

Twenty four phenolic standards were selected for preliminary qualitative analysis. MS detection was chosen to be the main assessment method for chemical identification and characterisation of the plant extracts because it provides the specific molecular mass of the different constituents in a sample and can confirm the molecular weight of a compound. Optimization of HPLC-MS and HPLC-PDA gradient systems are described in Chapter 2, section 2.4, and presented as a percentage of acetonitrile (%ACN) against time in Fig. 3.6. With acetonitrile and 0.1% aqueous acetic acid as mobile phase, all of the twenty four reference compounds could be identified with symmetric peak shapes and a short duration of analysis (see Appendix 1). Retention times (t_R), mass to charge ratios (m/z), ion modes of MS detection and maximum wavelengths (λ_{max}) of individual standards are presented in Table 3.4. Thus, identification of these standards was based on three independent criteria. There were slight differences in t_R between HPLC-MS and HPLC-PDA due to different HPLC equipment and column manufacturers (both column were of the same type). The HPLC-MS

system also had the facility to monitor UV absorbance at two wavelengths and values of 260 and 330 nm in HPLC-MS were selected based upon the λ_{max} obtained from HPLC-PDA. Likewise, the MS of each standard was operated in both negative and positive ion modes. To avoid a decrease in sensitivity from ion suppression and blockage of the MS detector, the concentration of the samples was limited to 2-20 ng per injection.

Table 3.4 Retention time (t_R), selected wavelengths (λ_{actual}), mass to charge ratios (m/z), ion modes of MS detections and maximum wavelengths (λ_{max}) of phenolic standards from HPLC-MS and HPLC-PDA analyses.

No.	Compound	HPLC-MS				HPLC-PDA	
		t_R (min)	λ_{actual} (nm)	m/z	Ion mode	t_R (min)	λ_{max} (nm)
1	Gallic acid	4.6	260	169	(-)ve	5.0	272.4
2	Caffeic acid	7.6	330	179	(-)ve	9.0	323.5
3	Vanillic acid	7.8	260	167	(-)ve	9.9	261.4
4	Rutin	9.1	260	609	(-)ve	11.8	256.8
5	Verbascoside	9.9	330	623	(-)ve	12.7	330.0
6	4-Coumaric acid	11.5	330	163	(-)ve	15.3	309.7
7	Ferulic acid	13.9	330	193	(-)ve	16.4	322.1
8	Rosemarinic acid	15.1	330	359	(-)ve	16.8	328.7
9	Naringin	16.2	260	579	(-)ve	16.3	284.2
10	Scutellarein	17.5	330	287	(+)ve	17.9	334.1
11	Luteolin	18.3	330	287	(+)ve	18.6	346.5
12	Nepetin	18.4	330	315	(-)ve	18.7	345.2
13	Quercetin	18.6	260	301	(-)ve	18.8	256.0
14	Cinnamic acid	19.3	260	147	(-)ve	19.6	281.7
15	Apigenin	19.3	330	271	(+)ve	19.8	334.3
16	Naringenin	19.4	260	271	(-)ve	19.7	290.6
17	Hispidulin	19.4	330	301	(+)ve	20.0	333.5
18	Kaempferol	19.5	260	287	(+)ve	20.0	out of scanning range
19	Chrysoeriol	19.6	330	299	(-)ve	20.1	344.6
20	Diosmetin	19.6	330	301	(+)ve	20.1	343.9
21	Hesperetin	19.8	330	301	(-)ve	20.1	289.0
22	Cirsimaritin	21.1	330	315	(+)ve	22.1	332.9
23	5,7-Dimethoxycoumarin	21.6	330	207	(+)ve	22.2	326.5
24	Chrysin	22.9	260	253	(-)ve	24.0	268.3

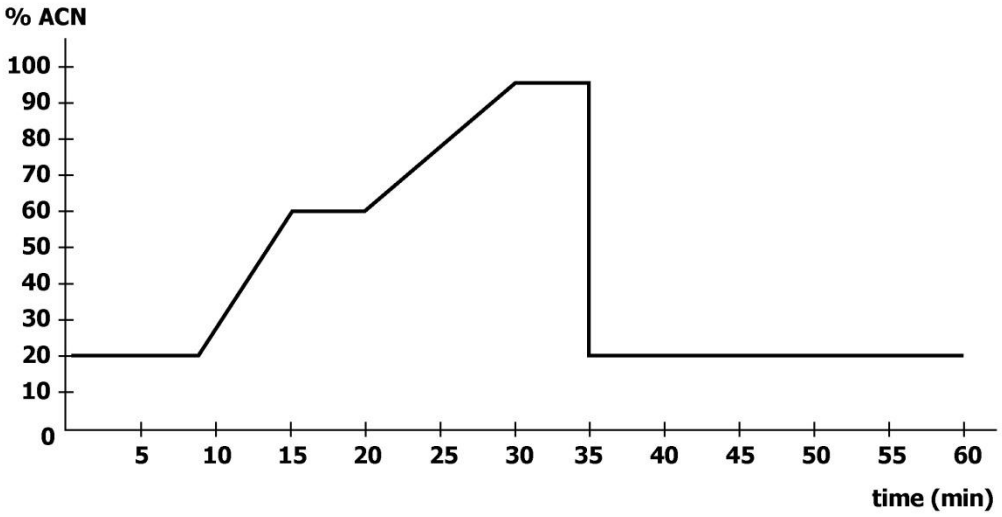


Fig. 3.6 Optimised method for HPLC-MS and HPLC-PDA.

From preliminary MS spectra of the plant samples, ethanolic extracts, the butanol fraction, and column chromatography fractions, eleven phenolic compounds were tentatively identified and selected as characteristic markers. Vanillic acid, verbascoside, 4-coumaric acid, ferulic acid, nepetin, naringenin, hesperetin and chrysin were detected in negative ion mode, whereas luteolin, apigenin, and hispidulin were detected in positive ion mode.

The linearity of the concentration versus peak area was determined over the range of 0.01-6 µM. The resulting fits and correlation coefficients (r^2) are shown in Table 3.5. Linear correlations were obtained with $r^2 > 0.95$ for all phenolic standards except hesperetin (r^2 = 0.90).

Based on a 20-µL injection, the limits of detection (LOD) and the limit of quantification (LOQ) for each standard were determined to be 20-25 nM and 41-50 nM, respectively for luteolin, apigenin, naringenin, hispidulin, hesperetin and chrysin. LODs of vanillic acid, 4-coumaric acid and ferulic acid were 32-38 nM and those of verbascoside and nepetin were 60 and 79 nM, respectively, whereas their LOQs were in the range of 63-80 nM except for nepetin (detected at 158 nM).

Table 3.5 Retention time (t_R); mass to charge ratios (m/z); ion modes; limit of detection (LOD; S/N ≥3); limit of quantification (LOQ; S/N ≥ 10); the square of the correlation coefficient (r^2); calibration curves (y = ax+b) of the eleven phenolic markers detected by MS detection (concentration range: 0.01-6 µM in methanol, n=3).

Compound	t_R (min)	m/z	Ion mode	LOD (nM)	LOQ (nM)	r^2	y = ax+b	
							a	b
Vanillic acid	8.0	167	(-)ve	37.2	74.3	0.98	81180	-1649
Verbascoside	10.1	623	(-)ve	60.0	80.1	0.96	104800	-9224
4-Coumaric acid	11.9	163	(-)ve	38.1	76.2	0.97	174700	-211
Ferulic acid	14.1	193	(-)ve	32.2	64.4	1.00	177600	-9771
Nepetin	18.4	315	(-)ve	79.1	158.1	0.95	519500	-94370
Luteolin	18.4	287	(+)ve	21.8	43.7	0.97	470200	10010
Apigenin	19.3	271	(+)ve	23.1	46.3	0.96	791500	59520
Naringenin	19.4	271	(-)ve	23.0	46.0	1.00	958700	-37970
Hispidulin	19.5	301	(+)ve	20.8	41.6	0.95	268400	-1880
Hesperetin	19.8	301	(-)ve	20.7	41.2	0.90	647800	9493
Chrysin	23.0	253	(-)ve	24.6	49.2	0.95	776700	78860

Multiple injections were carried out to determine the precision of the assay for each standard as shown in Table 3.6. The intra-day RSD (relative standard deviation) values at medium and high concentrations of each standard calibration curves were less than 5% for the eleven phenolic standards except for 4-coumaric acid (6.3%), while the intra-day RSDs at low concentration were relatively higher (2-19%). The inter-day RSD values were not significantly different among the low, medium and high concentrations.

Table 3.6 Intra- and inter-day precisions at low, medium, high concentrations of calibration curves of each phenolic standard with MS detection in their selected ion modes.

Compound	Ion mode	Intra-day (%RSD, n=5)			Inter-day (%RSD, n=6)		
		Low conc.	Medium conc.	High conc.	Low conc.	Medium conc.	High conc.
Vanillic acid	(-)ve	4.7	2.5	3.4	2.3	2.2	2.7
Verbascoside	(-)ve	4.9	2.7	2.3	2.5	1.7	1.4
4-Coumaric acid	(-)ve	12.0	6.3	2.6	17.1	13.7	14.1
Ferulic acid	(-)ve	19.1	1.6	1.4	8.3	2.7	4.7
Nepetin	(-)ve	8.1	2.2	1.8	6.9	2.2	2.8
Luteolin	(+)ve	2.0	1.4	5.0	17.0	18.1	15.2
Apigenin	(+)ve	8.8	3.6	4.0	13.2	8.7	13.6
Naringenin	(-)ve	3.0	1.9	1.8	2.9	1.8	1.7
Hispidulin	(+)ve	12.9	2.1	2.2	9.4	10.3	9.3
Hesperetin	(-)ve	6.5	2.7	4.6	18.4	10.6	8.1
Chrysin	(-)ve	3.1	0.6	1.0	3.0	2.1	0.6

Overall, the analytical parameters (r^2, LOD, LOQ, intra- and inter-day RSDs) showed that the optimised HPLC-MS method is sufficient and suitable to characterise the eleven potential phenolic compounds in the plant extracts.

3.2.4 Identification of chemical compounds in medicinal plants

To characterise naturally-occurring chemical compounds in *A. ebracteatus* (AE) and *C. petasites* (CP) extracts, direct injections at a concentration of 0.1 mg·mL^{-1} in methanol (20 µL) under the optimised HPLC-MS gradient system were performed and detected in negative and positive ion modes (Table 3.7 and Table 3.8). As well as confirming peak identities, PDA detection (a wavelength scanning range of 240-360 nm) was applied and provided maximum wavelengths (λ_{max}) of each characteristic peak to match with those of the eleven reference standards. NMR spectroscopy was also used to clarify and distinguish certain isomeric compounds. Therefore, there are four independent criteria corresponding with pure standards: (a) retention time (t_R), (b) mass to charge ratio (m/z), (c) maximum wavelengths (λ_{max}), and (d) NMR spectrum, to characterise unknown peaks. Chemicals with at least two criteria of identification were regarded as identified compounds in this study. It is noted that the retention time must be the first criteria to consider when identifying a compound matched with a standard. If retention times are different, the compound is certainly not the same as the standard tested.

Table 3.7 The characteristic peaks of ethanolic extracts of *A. ebracteatus* (0.1 mg·mL^{-1} in methanol) with MS detection in both negative and positive ion modes. Four independent criteria corresponding with pure standards: (a) retention time (t_R), (b) mass to charge ratio (m/z), (c) maximum wavelengths (λ_{max}), and (d) NMR spectra, were applied.

R_t (min)	m/z (-)ve	m/z (+)ve	λ_{max} (nm)	Identification	Number of independent criteria	Type of identification
6.2	-	476	-	Unknown	-	-
7.8	-	564	-	Unknown	-	-
8.0	167	-	261.4	Vanillic acid	3	a, b, c
10.1	623	-	330.0	Verbascoside	3	a, b, c
25.8	-	415	-	Unknown	-	-
34.5	-	458	-	Unknown	-	-

Table 3.8 The characteristic peaks of ethanolic extracts of *C. petasites* (0.1 mg·mL^{-1} in methanol) with MS detection in both negative and positive ion modes. Four independent criteria corresponding with pure standards: (a) retention time (t_R), (b) mass to charge ratio (m/z), (c) maximum wavelengths (λ_{max}), and (d) NMR spectra, were applied.

R_t (min)	m/z (-)ve	m/z (+)ve	λ_{max} (nm)	Identification	Number of independent criteria	Type of identification
6.2	-	476	-	Unknown	-	-
7.4	179	-	-	Caffeic acid*	2	a, b
7.8	-	564	-	Unknown	-	-
8.0	167	-	261.4	Vanillic acid	3	a, b, c
10.1	623	-	330.0	Verbascoside	3	a, b, c
11.9	163	-	-	4-Coumaric acid	2	a, b
14.1	193	-	322.1	Ferulic acid	3	a, b, c
18.4	-	287	-	Luteolin	2	a, b
18.5	315	317	345.2	Nepetin	3	a, b, c
19.3	-	271	333.4	Apigenin	3	a, b, c
19.4	271	-	-	Naringenin	2	a, b
19.6	299	301	333.5	Hispidulin	4	a, b, c, d
19.8	301	-	-	Hesperetin	2	a, b
20.5	-	297	-	Unknown	-	-
23.0	253	-	-	Chrysin	2	a, b
23.8	-	315	-	Unknown	-	-
25.8	-	415	-	Unknown	-	-
26.8	-	505	-	Unknown	-	-
34.5	-	458	-	Unknown	-	-

*not repeatable results

Two of six compounds in AE were identified as vanillic acid and verbascoside, whereas eleven compounds of CP were characterised as vanillic acid, verbascoside, 4-coumaric acid, ferulic acid, luteolin, nepetin, naringenin, hispidulin, hesperetin, and chrysin. Therefore, CP was chosen as the main plant candidate for quantitative analysis and further study with respect to skin permeation.

As far as the HPTLC results were concerned (Fig. 3.2-B), hispidulin was suspected to be a predominant compound in CP due to its quenching effect. It was also identified by HPLC-MS and with PDA detection. However, not only hispidulin has a molecular weight (MW) of 300 Da, there are also the two isomeric forms: chrysoeriol and diosmetin (Fig. 3.7). They share the same MW, polarity and chromophores, and thus they cannot be unambiguously identified by either MS or UV detectors. Therefore, NMR was used to elucidate these isomers. Although hispidulin has been previously reported (Hazekamp *et al.*, 2001; Klaiklay, 2009; Singharachai *et al.*, 2011a), none of this earlier work has unambiguously excluded the isomers by NMR spectroscopy.

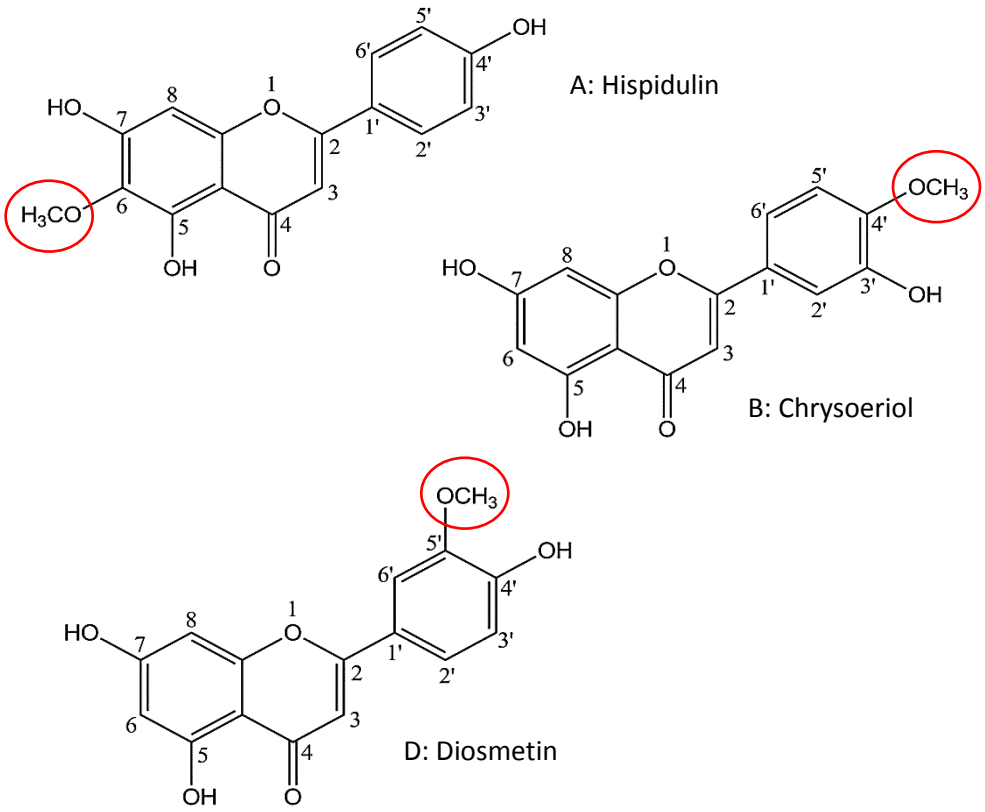


Fig. 3.7 Chemical structures of hispidulin, chrysoeriol and diosmetin.

NMR data obtained from the crude ethanolic extracts and the four fractional extracts from liquid-liquid partition were insufficiently pure to clarify the isomers. Column chromatography was therefore used to enrich and partially purify the samples for NMR detection. The eluent system was changed from 100% ethyl acetate to 100% methanol over time and each fraction was screened by HPLC-MS (Table 3.9). The fraction of pure ethyl acetate contained the highest amounts of a molecule with m/z 301 (suspected to be hispidulin). This fraction was then elucidated by NMR (^{1}H) to reveal peaks the chemical shifts of which clearly matched these of the hispidulin standard (Fig. 3.8).

Table 3.9 Concentrations of phenolic constituents of *C. petasites* fractions from column chromatography with MS detection; a single determination.

Compound	**Concentration of compound (μM)**								
		Ratio of methanol in a mixture solution of ethyl acetate							
	Initial	**0%**	**1%**	**2%**	**5%**	**10%**	**20%**	**50%**	**100%**
Vanillic acid	4.4	0.1	0.3	2.6	0.2	0.04	-	-	-
Verbascoside	1.6	-	-	-	-	-	-	-	0.04
4-Coumaric acid	0.2	0.1	0.1	0.1	-	-	-	-	-
Ferulic acid	-	-	0.04	0.2	-	-	-	-	-
Nepetin	13.8	-	-	-	0.1	0.1	0.3	2.8	0.6
Luteolin	0.4	-	0.01	-	-	-	-	0.1	0.01
Apigenin	1.2	2.3	1.5	0.1	0.1	0.02	-	-	-
Naringenin	-	0.3	0.03	-	-	-	-	-	-
Hispidulin	30.8	7.7	5.4	3.9	1.1	0.3	0.2	0.1	0.1
Hesperetin	0.6	0.1	0.1	0.1	0.02	0.01	-	-	-
Chrysin	-	0.1	-	-	-	-	-	-	-

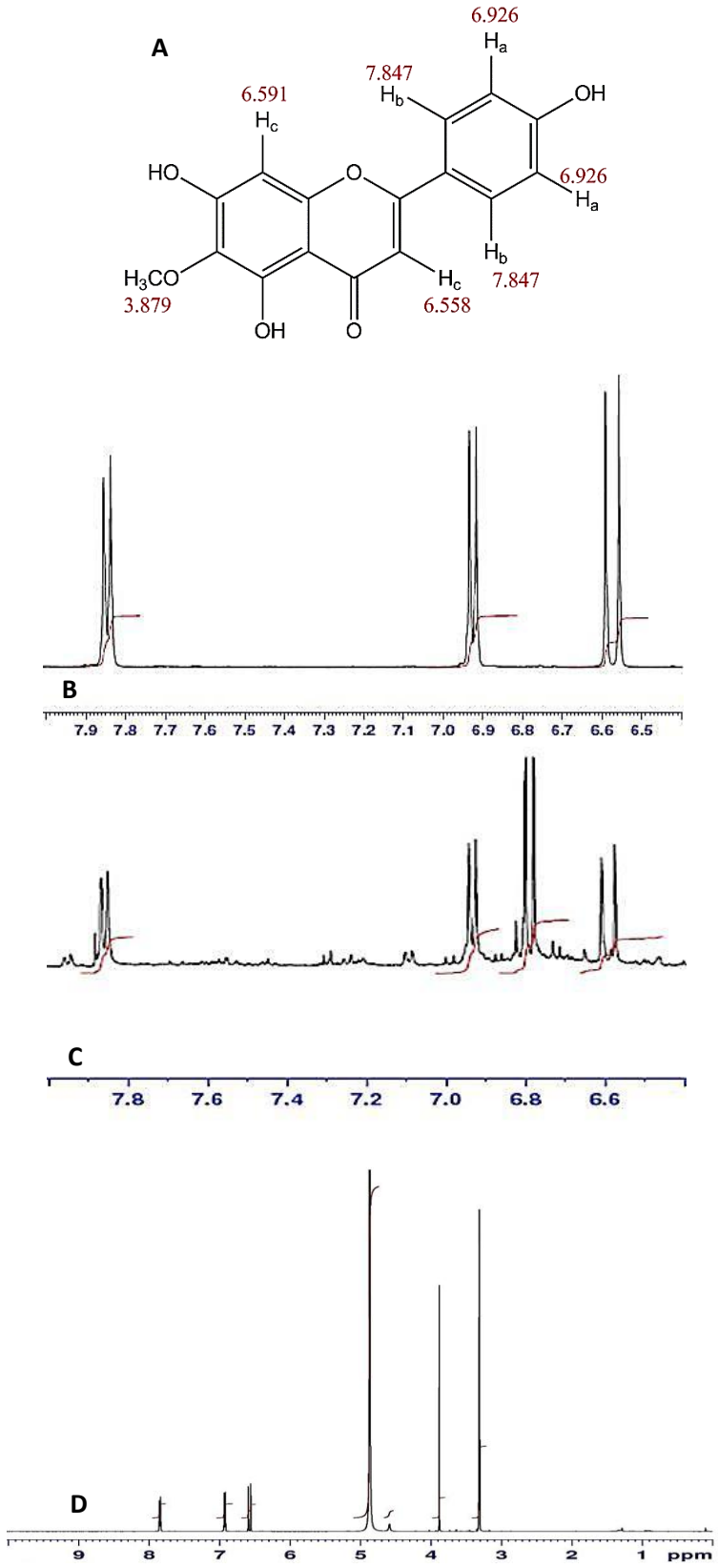


Fig. 3.8 NMR (^{1}H, 500 MHz in CD_3OD) of *C. petasites* compared to those of hispidulin standard: (A) chemical structure of hispidulin, (B) NMR of hispidulin standard, (C) NMR (range 6-8 ppm) of *C. petasites*, (D) full NMR (range 0-10 ppm) of hispidulin.

NMR proton analysis of the hispidulin standard from Fig. 3.8-D can be described as follows from right to left. The chemical shift at 3.879 ppm is the methoxyl group (C6 position). The peaks at 6.558 and 6.591 ppm are two singlets for protons H_c at C3 and C8. The doublets at 6.926 and 7.847 show para-disubstitution and are H_a (C3' and C5' positions) and H_b (C2' and C6' positions), respectively. As a result, the molecule with MW 300 Da in CP is confidently confirmed as hispidulin by four independent criteria of identification.

3.2.5 Quantification of natural constituents in the *C. petasites*

Amounts of phenolic compounds in CP (Table 3.10) were determined from direct injections of samples, ethanolic extracts, supercritical fluid extracts (SFE), butanol-fraction extracts, and powdered crude, into the HPLC-MS. Five components including vanillic acid, verbascoside, nepetin, apigenin and hispidulin, were quantified in the ethanolic extracts. The amounts of the other six compounds, 4-coumaric acid, ferulic acid, luteolin, naringenin, hesperetin and chrysin, were trace and fell below their LOQs. Hispidulin was predominant with 39 µmol/g followed by nepetin (15 µmol/g), verbascoside (4 µmol/g), vanillic acid (3 µmol/g) and apigenin (1 µmol/g), respectively. Reproducibility of the ethanolic extraction among five different batches was good.

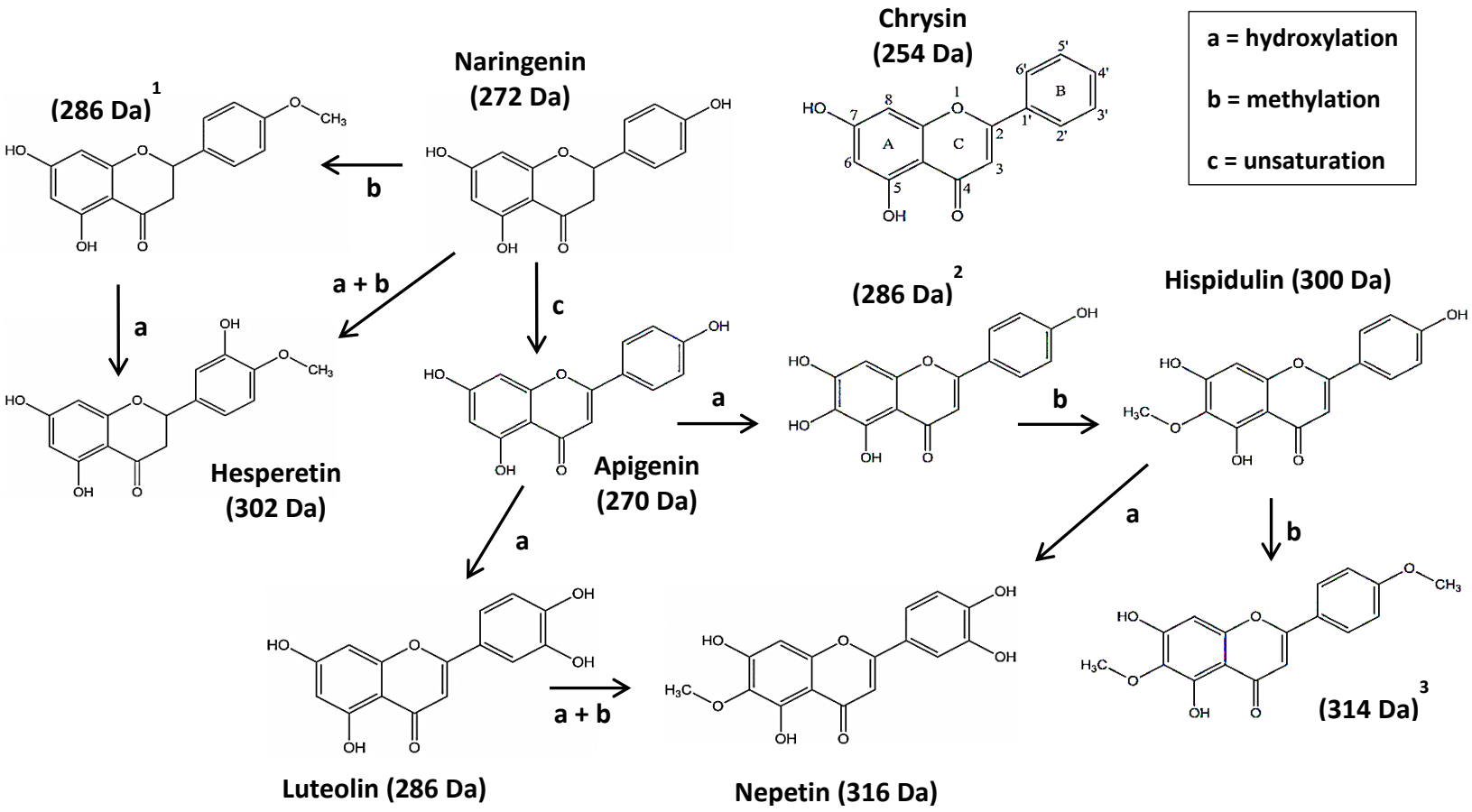
Injections of SFE and butanol-fraction extracts to compare signals and to confirm the presence of vanillic acid and verbascoside were undertaken. Vanillic acid was confirmed in SFE extracts with an increased amount of 12 µmol/g and verbascoside was highly present in butanol-fraction extracts (10 µmol/g). SFE and butanol-fraction extracts were used to reduce some aqueous-soluble interferences and non-polar artifacts, respectively, which may overshadow the peaks of interest. Without any extraction or separation, only nepetin and hispidulin (2 µmol/g) were detected in powdered crude plant material.

Table 3.10 Amounts of phenolic constituents in *C. petasites* from various extractions (0.1 $mg \cdot mL^{-1}$ in methanol) with the MS detection in both negative and positive ion modes; a single determination.

		Amount (μmol/g) in dried extract of *C. petasites*				
	Batch no.	**Vanillic acid**	**Verbascoside**	**Nepetin**	**Apigenin**	**Hispidulin**
Ethanolic	1	3.4	4.8	17.9	0.7	35.7
extracts	2	2.5	3.9	12.8	-	29.8
	3	3.7	5.3	17.4	1.2	49.3
	4	2.0	2.9	11.5	0.6	35.0
	5	2.0	2.5	12.8	1.0	42.5
	Average ± SD	*2.7 ± 0.8*	*3.9 ± 1.2*	*14.5 ± 2.9*	*0.9 ± 0.3*	*38.5 ± 7.6*
SFE extract		11.5	-	2.9	-	11.3
Butanol fraction		-	10.3	-	-	-
Crude dried plant material		-	-	2.1	-	1.5

3.2.6 Biosynthesis of naturally-occurring flavonoids in *C. petasites*

Seven phenolic compounds with the flavonoid skeleton including luteolin, nepetin, apigenin, naringenin, hispidulin, hesperetin, and chrysin (Table 3.8) were found in *C. petasites*. These arise via a network of biosynthetic reactions as shown in Fig. 3.9. In this scheme, naringenin with MW 272 Da is the "parent" of the flavonoid family. Unsaturation of the C ring yields the typical flavone apigenin. Further hydroxylation and methylation reactions have a broad specificity to yield the other compounds, sometimes by more than one possible pathway. Three unknown compounds believed to be flavonoids remain to be accounted for. A peak with MW 286 Da^1 could be an intermediate on the pathway to hesperetin and another peak with MW 286 Da^2 was shown not to correspond to scutellarein. It may be an intermediate between apigenin and hispidulin. Two peaks with MW 314 Da^3 may be methylation products of hispidulin, though cirsimaritin was ruled out by comparison with a standard. Chrysin, however, appears not to be on the main biosynthetic pathway to the other flavonoids.



1= structure of isosakuranetin; 2 = structure of scutellarein; 3 = structure of 6-methoxyacacetin

Fig. 3.9 Relation of naturally-occurring flavonoids in *C. petasites*.

3.3 Conclusions

The HPTLC fingerprints of *A. ebracteatus* and *C. petasites* were established using a mobile phase comprising ethyl acetate, methanol, water and formic acid in the ratio of 50:4:4:2.5 v/v/v/v. Ethanolic ferric (III) chloride spraying reagent was used to identify phenolic compounds whilst overspraying with H_2SO_4 revealed possible terpenoid constituents. The method is reproducible and efficient to distinguish between the two plants. Quality control of five batches was achieved and the procedures used were fast and simple.

With respect to the qualitative results of HPTLC, NMR and HPLC-MS, *C. petasites* was chosen as the main plant candidate because it contains greater numbers of phenolic compounds. Furthermore, a majority of chemical constituents in *A. ebracteatus* were glycosides which are less interesting in this study. Eleven phenolic compounds including vanillic acid, verbascoside, 4-coumaric acid, ferulic acid, nepetin, luteolin, apigenin, naringenin, hispidulin, hesperetin and chrysin, were found in *C. petasites*. Only five of them were present in a sufficient concentration to quantify. Hispidulin is a predominant compound (39 µmol/g) followed by nepetin, verbascoside, vanillic acid, and apigenin.

Chapter 4 *In vitro* percutaneous absorption of phenolic compounds in *C. petasites* from topical formulations – solution, paste, cream and lotion

4.1 Study purpose

In Thai traditional medicine, the most common preparation for skin treatment is a poultice. The ground plant of *C. petasites* is mixed with oils, such as coconut and sesame, or dispersed in alcohol, such as Thai rice whiskey, before application to the skin. Topical *C. petasites* products with reliability in quality, safety and efficacy have not been marketed. Only crushed leaves and powders are currently available. To optimise efficient topical formulations, *in vitro* studies of such products are required to determine the rate and extent of percutaneous absorption of the potentially active species.

The study aims are:

- To discover which naturally-occurring compounds in *C. petasites* are able to penetrate through the skin and to evaluate their permeability.
- To optimise topical formulations for *C. petasites* to achieve skin permeability of the active compounds.

4.2 Results and discussion

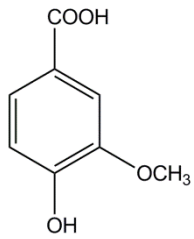
4.2.1 Prediction of maximum fluxes (J_{max}) of the phenolic reference compounds

The feasibility of topical absorption of the eleven naturally-occurring phenolic reference compounds was evaluated by predicting their maximum fluxes (J_{max}) (Table 4.1) from their physicochemical properties: molecular weight (MW), octanol-water partition coefficient (log P), and water solubility ($C_{sat,w}$) (ALOGPS 2.1 algorithm, 2001; Chemspider). Fig. 4.1 shows the chemical structures of these compounds to complement the explanation of their permeability.

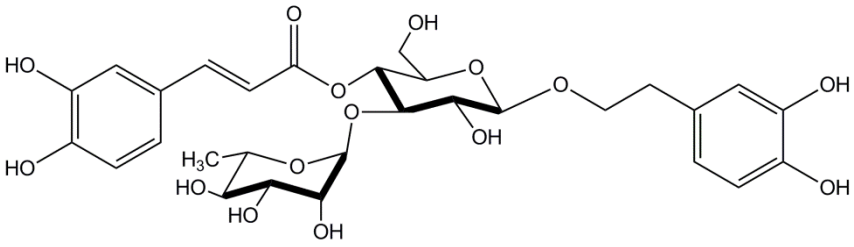
Table 4.1 Physicochemical properties (molecular weight (MW), octanol-water partition coefficient (log P), and water solubility ($C_{sat,w}$)) of the eleven phenolic compounds and their predicted maximum permeation rate (J_{max}).

Compound	MW (Da)	log P	$C_{sat,w}$ [b] (mM)	Predicted J_{max} ($nmol \cdot cm^{-2} \cdot h^{-1}$)
Vanillic acid	168.2	1.4[a]	38.0	73.3
Verbascoside	624.6	-0.03 ± 1.0[b]	1.5	0.0005
4-Coumaric acid	164.2	1.5[a]	11.0	23.5
Ferulic acid	194.2	1.5[a]	9.5	14.6
Nepetin	316.3	2.0 ± 0.8[b]	0.4	0.2
Luteolin	286.2	2.5[a]	0.6	1.3
Apigenin	270.2	2.3 ± 0.6[b]	0.8	1.5
Naringenin	272.3	2.5[a]	1.1	3.0
Hispidulin	300.3	2.2 ± 0.7[b]	0.4	0.4
Hesperetin	302.3	2.1[a]	0.8	0.7
Chrysin	254.2	3.5[a]	0.5	8.0

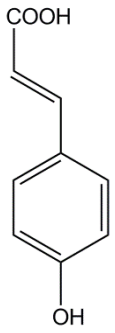
a = experimental value; b = predicted value



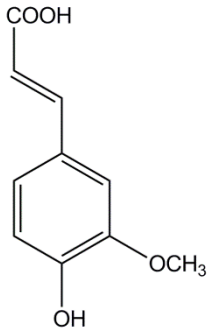
Vanillic acid



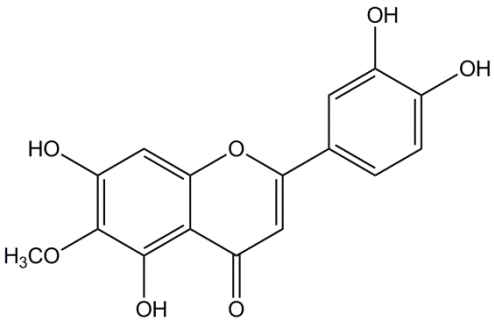
Verbascoside



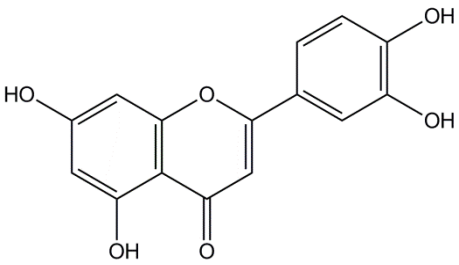
4-Coumaric acid



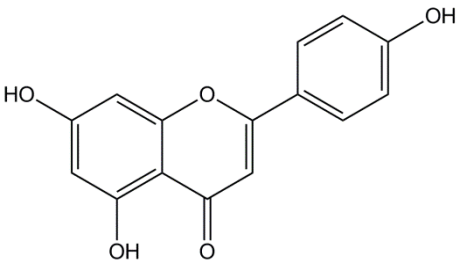
Ferulic acid



Nepetin



Luteolin



Apigenin

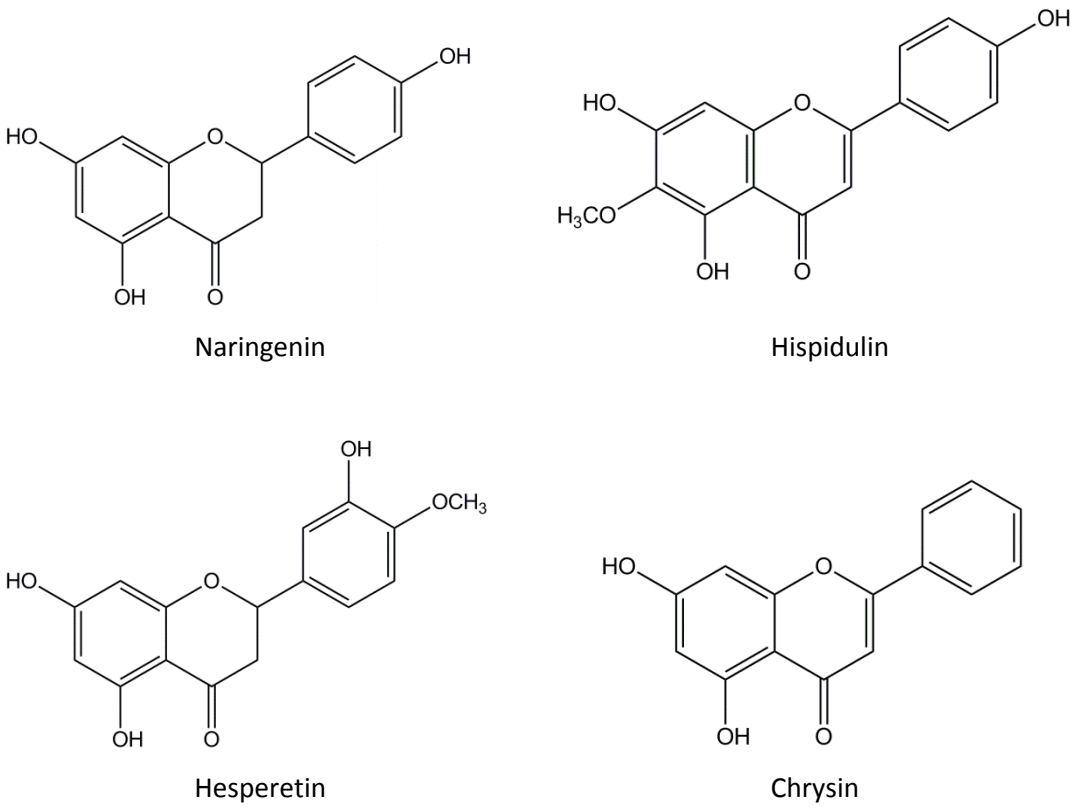


Fig. 4.1 Chemical structures of the eleven phenolic compounds.

The eleven compounds may be broadly categorised into three groups: phenolic acids, flavonoid aglycones, and a phenolic glycoside. Phenolic acids including vanillic acid, 4-coumaric acid and ferulic acid; their predicted J_{max} are highest because of their smaller size and greater water solubility. Flavonoid aglycones, nepetin, luteolin, apigenin, naringenin, hispidulin, hesperetin, and chrysin, have slightly larger MW but are less soluble in water than the phenolic acids; hence, their predicted penetration rates are slower. Verbascoside is the only phenolic glycoside in this study and contains two sugars, rhamnose and β-glucose; it has the highest MW as a result and a lower log P which (despite its reasonable water solubility) means that this compound has the lowest predicted J_{max}.

Overall, ten of the eleven compounds have predicted fluxes of at least ~0.1 $\mu g \cdot cm^{-2} \cdot h^{-1}$; only verbascoside flux is unlikely to ever be of therapeutic use. The phenolic acids have physicochemical properties consistent with a reasonable skin permeability, modest MW and log P in the range of 1-3 (Hadgraft and Somers, 1956).

4.2.2 *In vitro* percutaneous absorption of the phenolic compounds from a CP solution and from a paste

C. petasites was selected as the principal plant for this study due to the wide range of polyphenolic compounds that it contains (detail in Chapter 3). Ethanolic extracts were initially formulated in paste and solution vehicles as models for Thai traditional preparations based on natural oils and alcohol. From a pharmaceutical view point, a paste can contain the highest amount of a powdered plant (up to 50% of the total recipe) whereas a solution is the dosage form that might be manipulated most easily to achieve the maximum fluxes of the ingredients. 50% aqueous ethanol was used to redissolve ethanolic plant extracts and was considered to be an acceptable donor vehicle for subsequent *in vitro* diffusion cell experiments. *In vitro* experiments with plant formulations (50% w/w CP paste and a 50 $mg \cdot mL^{-1}$ CP solution in 50:50 v/v ethanol:water) were performed using Franz diffusion cells and excised abdominal pig skin over a 24 hour period. Samples were withdrawn from the receptor solution at 3, 6 and 24 hours. The skin samples were subsequently subjected to tape stripping. Control experiments without any formulations were conducted. All receptor solution samples and tape-strip extractions were analysed by HPLC-MS, and the results expressed as the molar amount of compound detected per square centimetre of skin. None of the compounds was detected in the control experiments.

Table 4.2 Quantities of the eleven phenolic compounds detected in the receptor solution after 3, 6 and 24 hours.

Compound	**Quantity in receptor solution (average ± SD, $nmol \cdot cm^{-2}$, n=6)**					
	50% w/w CP paste			**50 $mg \cdot mL^{-1}$ CP solution (ethanol/water; 50:50)**		
	3 h	**6 h**	**24 h**	**3 h**	**6 h**	**24 h**
Vanillic acid	0.1 ± 0.07	0.5 ± 0.3	4.4 ± 2.9	-	0.4 ± 0.2	8.0 ± 3.7
Verbascoside	-	-	-	-	-	0.2 ± 0.1
4-Coumaric acid	-	-	0.2 ± 0.2	-	-	0.5 ± 0.2
Ferulic acid	-	-	0.3 ± 0.3	-	-	1.1 ± 0.6
Nepetin	0.1 ± 0.02	0.1 ± 0.03	0.4 ± 0.2	-	0.03 ± 0.01	3.2 ± 1.6
Luteolin	-	-	0.05 ± 0.02	-	-	0.3 ± 0.1
Apigenin	-	-	0.1 ± 0.1	-	-	0.9 ± 0.2
Naringenin	-	-	-	-	-	0.01 ± 0.002
Hispidulin	0.2 ± 0.2	0.7 ± 0.5	4.0 ± 2.4	0.1 ± 0.1	1.5 ± 0.8	21.4 ± 3.7
Hesperetin	-	-	0.2 ± 0.1	-	-	0.3 ± 0.1
Chrysin	-	-	-	-	-	-

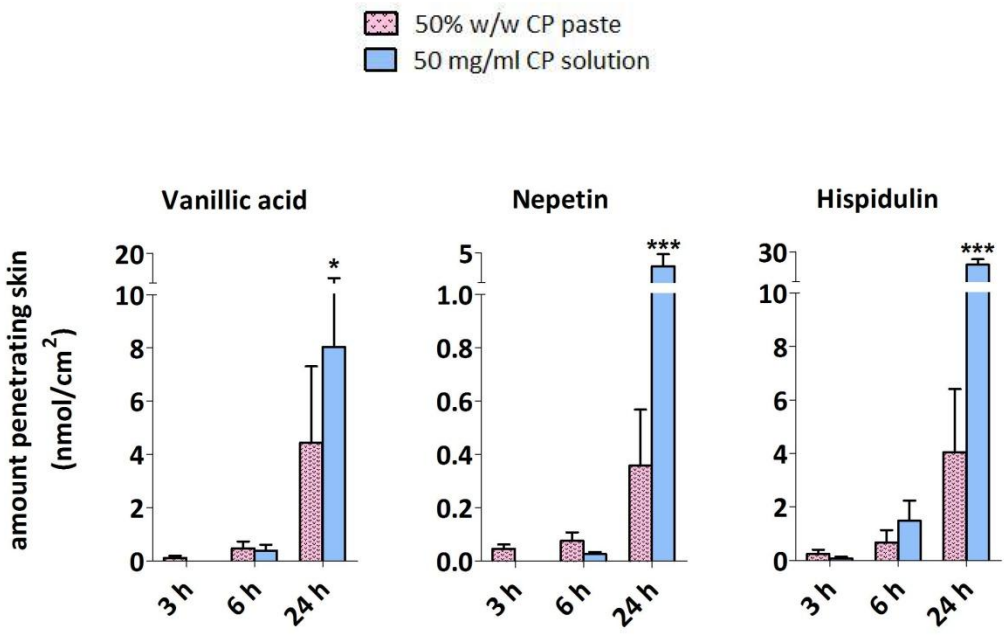


Fig. 4.2 Quantities (mean ± SD) of vanillic acid, nepetin, and hispidulin penetrated across the skin at 3, 6 and 24 hours following application of either 50% w/w CP paste or an ethanol:water CP solution (50 mg·mL^{-1}).

Hispidulin was the only compound that penetrated from both formulations through the skin in an amount that could be quantified after 3 hours (Table 4.2 and Fig. 4.2). While both vanillic acid and nepetin applied as a CP solution were detectable in the receptor phase at 3 hours, they could not be quantified because of their relatively high LOQs (74 and 158 mM, respectively) compared to that of hispidulin (42 mM).

As expected, verbascoside delivered from the CP paste was not detected in the receptor solution after 24 hours because it is not soluble in the fatty base of this formulation. Hence, even though CP has a relatively high verbascoside content (4 µmol/g), its insolubility in the paste prevented any percutaneous absorption to be measured. However, verbascoside was detected when delivered from the hydroalcoholic solution, reflecting a better solubility. Indeed, just about all of the CP constituents appeared to be better absorbed from this vehicle.

Direct comparison of the theoretically predicted J_{max} values in Table 4.1 with the experimental data in Table 4.2 is not possible because the degrees of saturation of the different compounds in the two formulations are unknown. However, from a qualitative standpoint, it is perhaps reassuring to observe that verbascoside was expected to penetrate the skin poorly and this was indeed the case. Equally, vanillic acid was well-

absorbed and this was consistent with the relatively high J_{max} predicted from the model; nonetheless, the measured penetration of this compound was well below that anticipated from J_{max} suggesting that vanillic acid was present in the formulations at levels much less than the saturation concentration. The same is almost certainly true for 4-coumaric acid, ferulic acid, and chrysin, for which no detectable skin penetration was found. Interestingly, the predicted J_{max} values of nepetin and hispidulin would crudely suggest absorptions of 4.8 and 9.6 nmol·cm^{-2}, respectively, in 24 hours, values not that different from those observed experimentally (and suggesting, therefore, that these constituents were close to saturation in the vehicles).

Table 4.3 Percentage penetration of vanillic acid, verbascoside, nepetin, apigenin and hispidulin following applications of 50% w/w CP paste and 50 mg·mL^{-1} CP solution after 3, 6 and 24 hours. Values were determined from the ratio of the cumulative amount of compound in the receptor solution to its original content in the formulation applied.

Compound	**% penetration (average ± SD, n=6)**					
	50% w/w CP paste			**50 mg·mL^{-1} CP solution (ethanol/water; 50:50)**		
	3 h	**6 h**	**24 h**	**3 h**	**6 h**	**24 h**
Vanillic acid	0.1 ± 0.04	0.3 ± 0.2	2.9 ± 1.9	-	0.5 ± 0.3	10.6 ± 4.9
Verbascoside	-	-	-	-	-	0.1 ± 0.1
Nepetin	0.01 ± 0.002	0.01 ± 0.004	0.04 ± 0.03	-	0.01 ± 0.001	0.8 ± 0.4
Apigenin	-	-	0.2 ± 0.1	-	-	4.4 ± 1.0
Hispidulin	0.01 ± 0.01	0.03 ± 0.02	0.2 ± 0.1	0.01 ± 0.01	0.1 ± 0.1	2.0 ± 0.3

The percentages of the chemical amounts absorbed across the skin from the two formulations are presented in Table 4.3. These values are consistent with the perception that penetration from the hydroalcoholic solution was greater.

Table 4.4 Amounts per unit area of the eleven phenolic compounds in the stratum corneum (SC) 24 hours post-application of two formulations.

Compound	**Amount in SC after 24 hours (average ± SD, nmol·cm^{-2}, n=6)**	
	50% w/w CP paste	**50 mg·mL^{-1} CP solution (ethanol/water; 50:50)**
Vanillic acid	0.3 ± 0.1	0.2 ± 0.1
Verbascoside	0.4 ± 0.2	0.8 ± 0.1
4-Coumaric acid	-	-
Ferulic acid	-	-
Nepetin	1.1 ± 0.3	1.6 ± 0.6
Luteolin	-	0.6 ± 0.3
Apigenin	-	0.6 ± 0.3
Naringenin	-	-
Hispidulin	0.3 ± 0.01[a]	20.2 ± 8.4
Hesperetin	-	0.1 ± 0.04
Chrysin	-	-

a = only 3 replicates were measurable.

Supporting results from tape stripping experiments at 24 hours are shown in Table 4.4 and Fig. 4.3 (left panel) where they are compared with the penetration profiles from the Franz cell experiments (right panel). Hispidulin not only penetrated the skin well, it was also taken up significantly into the SC. Vanillic acid, while absorbed reasonably from both formulations, was not present in high levels in the SC, presumably a reflection of its less lipophilic character. Verbascoside, as anticipated, was unable to penetrate much beyond the most superficial layers of the SC. As for skin penetration, SC uptake of most compounds was greater from the ethanol:water solution than from the paste.

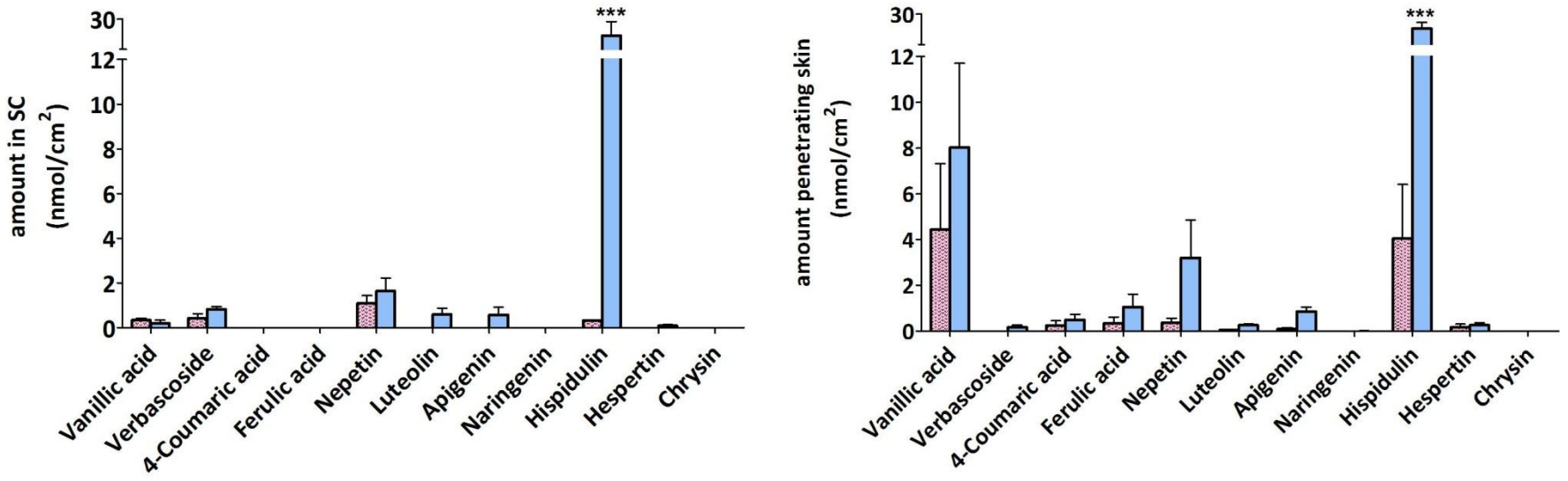


Fig. 4.3 Quantities (average ± SD) of the eleven phenolic compounds penetrated across the skin (right panel) and in the SC tape-strips at 24 hours. Dotted pink bars and solid blue bars indicate the applications of 50% w/w CP paste and an ethanol:water CP solution (50 $mg \cdot mL^{-1}$), respectively.

4.2.3 *In vitro* percutaneous absorption of the phenolic compounds from a CP cream and a lotion

Based on results from the CP paste and the ethanol:water CP solution, it was decided to select four compounds, vanillic acid, verbascoside, nepetin and hispidulin, for further study with a 10% w/w CP oil-in-water cream and a lotion.

Vanillic acid, nepetin and hispidulin were the three compounds showing the most substantial absorption. They were therefore the likeliest candidates for eliciting CP's pharmacological effect. Verbascoside, on the other hand, was chosen because of its apparent affinity to the SC and because it has a different structure and physicochemical properties that make it a poor permeant (i.e., a sort of a negative control for the other three compounds). Moreover, verbascoside with poor permeability through the SC can be at least beneficial for an antimicrobial activity against *Straphylococcus aureus*, *Enterococcus faecalis*, *Escherichia coli*, and *Psedomonas aeruginosa* (Shikanga *et al.*, 2010). The other possible bioactivities are an antioxidant activity via a radical scavenging activity (Sinico *et al.*, 2008), an anti-inflammatory activity by inhibiting the activation of nuclear factor-kappa B cells (NF-κB) (Esposito *et al.*, 2010), suppressing NF-κB and activator protein-1-deoxyribonucleic acid (AP-1-DNA) binding activity (Pastore *et al.*, 2009), and inhibiting lipid peroxidation (Korkina *et al.*, 2007), and a whitening activity by inhibiting tyrosinase activity and melanin production (He *et al.*, 2011).

The four compounds were examined in cream and lotion vehicles (i.e., more cosmetically acceptable than paste and ethanol:water solution). Non-ionic surfactants, Tween 60 and Span 60, were used to stabilise the formulations. The concentration of *C. petasites* was controlled at 10% by weight which has been reported as the maximal concentration of plant ethanolic extracts in topical products without measurable toxicity (Diwan *et al.*, 2001).

Equivalent sets of sample and control experiments were conducted with the same method as the *in vitro* studies described in section 4.2.2 but the application period was only 6 hours to allow comparison with the results of *in vivo* studies carried out in human volunteers later.

Table 4.5 Quantities per unit area of vanillic acid, verbascoside, nepetin and hispidulin detected in SC and in the receptor solutions after 6 hours.

Compound	Quantity after 6 hours (average ± SD, nmol·cm^{-2}, n=6)			
	10% w/w CP cream		10% w/w CP lotion	
	In SC	In receptor	In SC	In receptor
Vanillic acid	0.6 ± 0.3	0.6 ± 0.5	0.6 ± 0.2	0.4 ± 0.3
Verbascoside	0.3 ± 0.02[a]	-	0.5 ± 0.03[a]	-
Nepetin	0.8 ± 0.6	0.1 ± 0.02	0.9 ± 0.6	0.1 ± 0.03
Hispidulin	3.0 ± 0.4	1.1 ± 0.8	2.4 ± 0.4	0.7 ± 0.3

a = only 3 replicates were measurable.

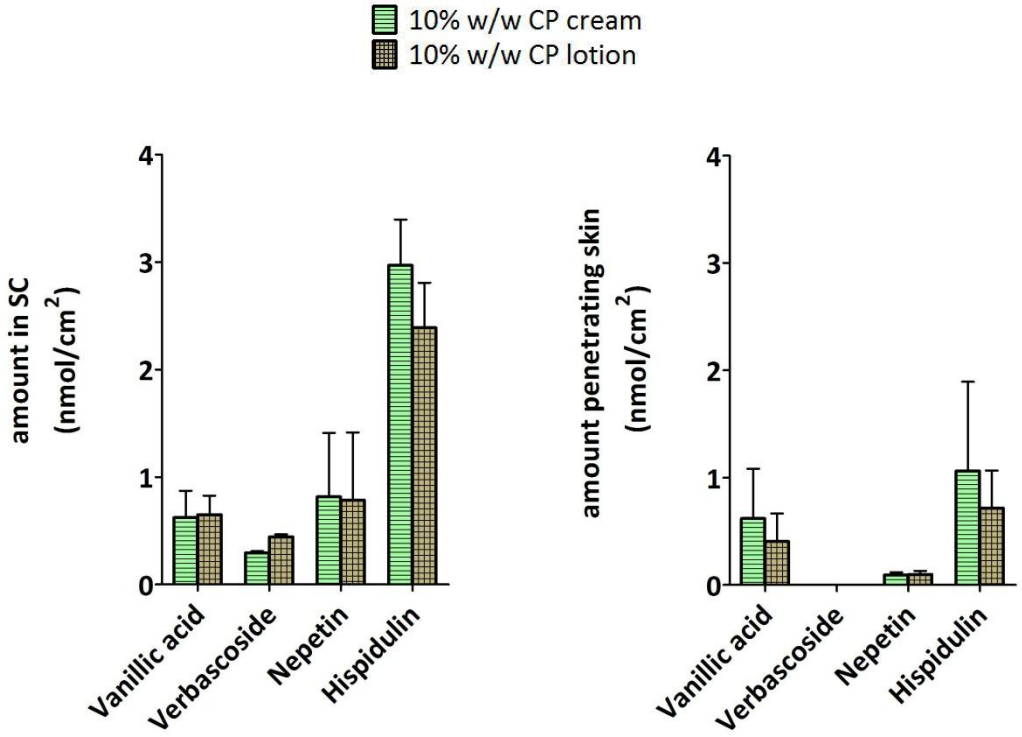


Fig. 4.4 Quantities (average ± SD) of vanillic acid, verbascoside, nepetin, hispidulin penetrated across the skin (right panel) and in the SC tape-strips (left panel) at 6 hours. Striped green bars and hatched brown bars indicate the applications of 10% w/w CP cream and 10% w/w CP lotion, respectively.

As expected, all compounds except verbascoside delivered from both cream and lotion vehicles were detected in the receptor phase after 6 hours (Table 4.5 and Fig. 4.4, right panel). Results from tape-strips (Table 4.5 and Fig. 4.4, left panel) also support the compounds' permeation in the Franz cell experiments. Vanillic acid and hispidulin were taken up into the SC and crossed the skin well. Verbascoside again was only distributed in the SC and was not percutaneously absorbed. Little difference was observed between the two formulations, the excipients of which were similar (Table 2.1, Chapter 2).

As explained in section 4.2.2, the theoretically predicted J_{max} values in Table 4.1 can be qualitatively compared with the experimental data in Table 4.5. Verbascoside's poor permeability was as anticipated. Vanillic acid was indeed well-absorbed but its penetration was much lower than that expected from the predicted J_{max}. The formulations presumably contained vanillic acid at much less than the saturation levels, therefore. The same assumption seems to apply to nepetin and hispidulin, the experimental absorption of which was lower than the predicted J_{max} (1.2 and 2.4 nmol·cm^2, respectively).

Table 4.6 Percentage penetration of vanillic acid, verbascoside, nepetin and hispidulin from the 10% w/w CP cream and the 10% w/w CP lotion after 6 hours. Values were determined from the ratio of the cumulative amount of compound in the receptor solution to its original content in the formulation applied.

Compound	**% penetration (average ± SD, n=6)**	
	10% w/w CP cream	**10% w/w CP lotion**
Vanillic acid	2.1 ± 1.5	1.3 ± 0.9
Verbascoside	-	-
Nepetin	0.1 ± 0.01	0.1 ± 0.02
Hispidulin	0.2 ± 0.2	0.2 ± 0.1

The percentage penetration in 6 hours of the four compounds from the CP cream and lotion (Table 4.6) was greater than that from the ethanol:water solution. This may be due to the presence of surfactants in the cream and lotion formulations facilitating the solubilisation of the compounds in the SC.

4.3 Conclusions

Of the eleven phenolic compounds identified in *C. petasites*, four compounds, vanillic acid, verbascoside, nepetin and hispidulin, were chosen for further topical delivery studies. Hispidulin was percutaneously absorbed within 3 hours and vanillic acid and nepetin permeated the skin after 6 hours. However, verbascoside remained in the superficial layers of the SC due to its large molecular size and high polarity. *In vitro* experiments were optimised to inform the design of a subsequent study in human volunteers. As the CP cream and lotion were more (and equally) efficient in delivering the four compounds across the skin (than the simple ethanol:water solution), these two formulations were selected for further investigation *in vivo*.

Chapter 5 *In vivo* assessment of a topical cream and a lotion containing 10% w/w *C. petasites*

5.1 Study purpose

In the *in vitro* permeation studies using pig skin, four phenolic compounds, vanillic acid, verbascoside, nepetin and hispidulin, were identified as relevant "actives" for topical delivery from *C. petasites* formulations. The cream and the lotion were selected for this *in vivo* study.

The study aims are:

- To identify and quantify naturally-occurring molecules in the SC after applying a cream and a lotion containing 10% w/w *C. petasites* to human skin *in vivo* for 6 hours.
- To compare the results with data from *in vitro* experiments.
- To determine whether the administration of the cream and lotion induced any adverse effect such as skin irritation.

5.2 Results and discussion

5.2.1 Analytical chemistry

HPLC-PDA was the principal analytical technique used in this study. Method optimisation and validation were performed. To increase sensitivity, tape stripped extracts were lyophilised, then redissolved in $1/10^{th}$ volume of solvent prior to HPLC-PDA analysis. Retention time (t_R) and maximum wavelength (λ_{max}), corresponding to those of pure standards, were the criteria for identification (Table 5.1).

The linearity of the detection was excellent ($r^2 > 0.99$ for all compounds over the concentration range of 0.08-600 µM). Based on a 20-µL injection, the limits of detection (LODs) and LOQs of hispidulin and nepetin (< 0.2 mM and < 0.8 mM, respectively) were

much lower than those of vanillic acid and verbascoside (LODs > 1.2 mM and LOQs > 1.6 mM).

Table 5.1 Retention time (t_R), maximum wavelength (λ_{max}), selected wavelength (λ_{actual}), limit of detection (LOD; S/N ≥ 3), limit of quantification (LOQ; S/N ≥ 10), the square of the correlation coefficient (r^2), and calibration curve parameters (y = ax+b) of vanillic acid, verbascoside, nepetin and hispidulin, detected by PDA (concentration range 0.08-600 µM in methanol, n= 3).

Compound	t_R (min)	λ_{max} (nm)	λ_{actual} (nm)	LOD (mM)	LOQ (mM)	r^2	y = ax+b	
							a	b
Vanillic acid	10.0	261.4	260	1.49	2.38	0.99	0.43 ± 0.01	-1.66 ± 1.42
Verbascoside	13.0	330.0	330	1.20	1.60	0.99	0.48 ± 0.01	0.02 ± 0.73
Nepetin	18.8	345.2	330	0.16	0.79	0.99	0.86 ± 0.01	-2.52 ± 1.23
Hispidulin	20.1	333.5	330	0.08	0.17	0.99	0.82 ± 0.01	-1.22 ± 1.43

The precision of the assay for each standard was determined at low, medium and high concentrations (Table 5.2). Relative standard deviations (RSD) of intra-day precision of all standards were less than 3% except for verbascoside (6.3% at low concentration). The inter-day RSD values were less than 5.3% for all compounds.

Table 5.2 Intra- and inter-day precision at low, medium, high concentrations of each phenolic marker.

Compound	t_R (min)	Intra-day (%RSD, n=5)			Inter-day (%RSD, n=6)		
		Low conc.	Medium conc.	High conc.	Low conc.	Medium conc.	High conc.
Vanillic acid	10.0	1.4	0.3	1.6	2.0	1.4	1.6
Verbascoside	13.0	6.3	2.1	2.2	5.2	2.8	5.3
Nepetin	18.8	2.4	0.5	1.7	3.2	2.8	2.2
Hispidulin	20.1	1.9	0.6	3.0	2.5	1.8	2.3

It follows that the optimised HPLC-PDA method provided a precise and accurate quantification of the four compounds.

5.2.2 *In vivo* permeation profiles of four compounds from a 10% w/w CP cream and a 10% w/w CP lotion

The formulations were separately applied to the ventral forearms of six volunteers and left for 6 hours before removal and tape stripping. Cumulative amounts of vanillic acid, nepetin and hispidulin taken up into the SC of the individual volunteers from the cream and lotion, respectively, are presented in Table 5.3 and Table 5.4. Transport of hispidulin from the cream (1 nmol·cm^{-2}) was approximately double that of nepetin and vanillic acid (0.4, and

0.3 nmol·cm^{-2}, respectively). Penetration of the compounds from the lotion (0.9, 0.5, and 0.4 nmol·cm^{-2} for hispidulin, nepetin and vanillic acid, respectively) were similar to those from the cream. Verbascoside was not detected in the SC due, presumably, to a combination of its low permeability and the reduced sensitivity of the PDA detection used. Reproducibility was good and the robustness of the *in vivo* methodology was shown by low inter-subject variability: less than 29% for hispidulin, 44% for vanillic acid, and 55% for nepetin.

Table 5.3 Amounts of vanillic acid, verbascoside, nepetin and hispidulin taken up into the SC after a 6-hour application of a 10% w/w CP cream to human volunteers.

Volunteer	**Amount in SC 6-hour post application of 10% w/w CP cream (average ± SD, nmol·cm^{-2}, n=3)**			
number	**Vanillic acid**	**Verbascoside**	**Nepetin**	**Hispidulin**
1	0.3 ± 0.1	-	0.4 ± 0.1	0.7 ± 0.1
2	0.3 ± 0.1	-	0.5 ± 0.2	1.1 ± 0.1
3	0.4 ± 0.1	-	0.6 ± 0.1	1.3 ± 0.2
4	0.2 ± 0.02	-	0.6 ± 0.003	0.9 ± 0.03
5	0.2 ± 0.04	-	0.6 ± 0.1	0.8 ± 0.1
6	0.6 ± 0.1[a]	-	0.1 ± 0.01[a]	1.1 ± 0.1[a]
Average	***0.3 ± 0.1***	***-***	***0.4 ± 0.2***	***1.0 ± 0.2***
%RSD	***43.1***	***-***	***48.5***	***23.1***

a= measurement by MS detection

Table 5.4 Amounts of vanillic acid, verbascoside, nepetin and hispidulin taken up into the SC after a 6-hour application of a 10% w/w CP lotion to human volunteers.

Volunteer	**Amount in SC 6-hour post application of 10% w/w CP lotion (average ± SD, nmol·cm^{-2}, n=3)**			
number	**Vanillic acid**	**Verbascoside**	**Nepetin**	**Hispidulin**
1	0.5 ± 0.1	-	0.4 ± 0.02	0.7 ± 0.2
2	0.4 ± 0.1	-	0.6 ± 0.1	1.2 ± 0.2
3	0.4 ± 0.04	-	0.6 ± 0.2	1.1 ± 0.1
4	0.2 ± 0.03	-	0.6 ± 0.1	0.9 ± 0.1
5	0.3 ± 0.1[a]	-	0.4 ± 0.3[a]	0.6 ± 0.1[a]
6	0.5 ± 0.1[b]	-	0.05 ± 0.02[b]	0.8 ± 0.2[b]
Average	***0.4 ± 0.1***	***-***	***0.5 ± 0.3***	***0.9 ± 0.2***
%RSD	***29.0***	***-***	***54.9***	***28.5***

a= 2 sites were measured by UV detection, a third by MS; b = 3 sites were measured by MS detection

Fig. 5.1 A-C compares the cumulative amounts of vanillic acid, nepetin and hispidulin absorbed from the two topical formulations for each volunteer. The SC uptake of each compound from the two vehicles was very consistent in each subject (only one statistically significant difference for vanillic acid in volunteer 1). There were inter-subject differences, possibly reflecting the variability in SC thickness between different individuals (see Fig. 5.1 D). When nepetin was detected by MS in volunteer 6, the levels observed were clearly the

smallest. While there should be little or no difference between the PDA and MS assays, it is possible that the PDA peak for nepetin was subject to interference by other compounds in the plant extract with a similar retention time (e.g., luteolin). It is possible, therefore, that the nepetin quantities found in volunteers 1-5 using PDA may over-estimate the actual amounts present in the SC. In contrast, no interfering peaks with those of vanillic acid and hispidulin were apparent.

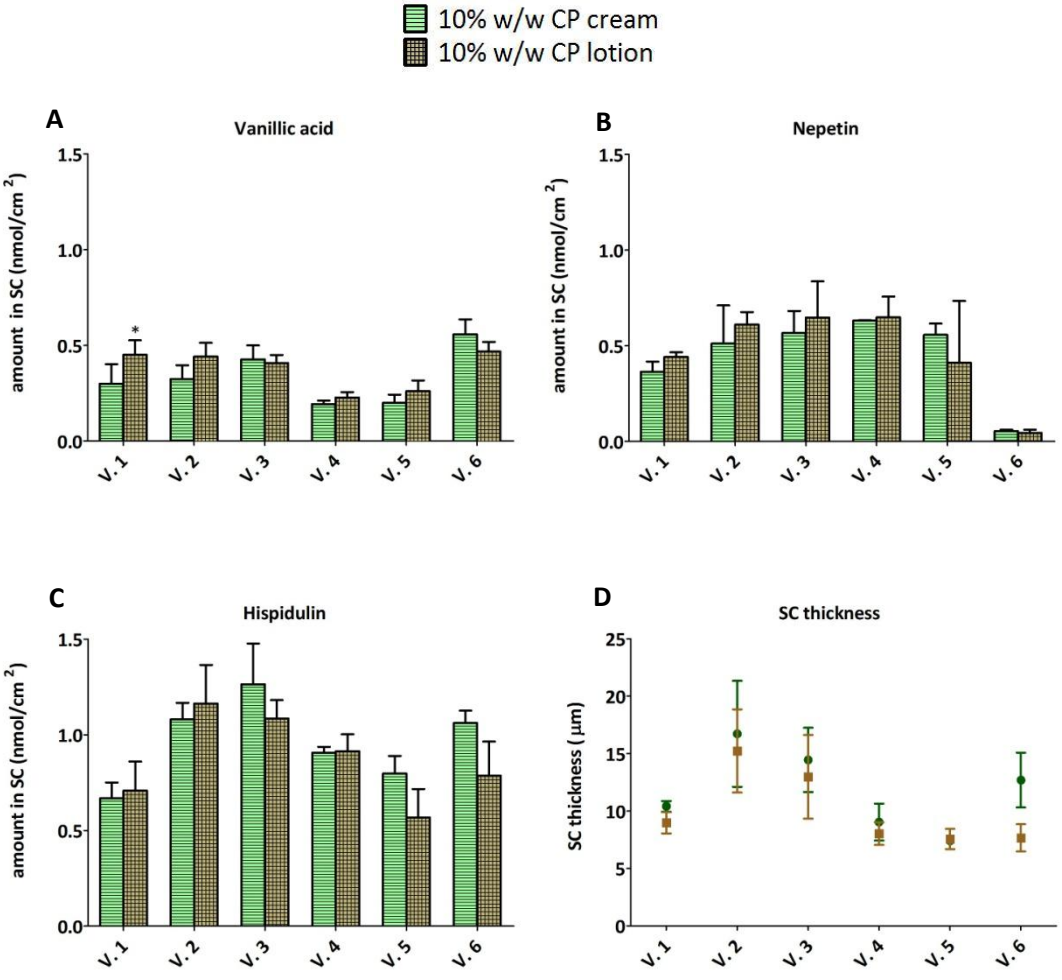


Fig. 5.1 A-C: Amounts (mean ± SD, n = 3) of vanillic acid, nepetin and hispidulin taken up into human SC *in vivo* after 6 hours; D: SC thickness was determined gravimetrically for each volunteer. Green bars (Figs. A-C) and green circles (Fig. D) correspond to application of a 10% w/w CP cream; brown bars (Figs. A-C) and brown squares (Fig. D) correspond to a 10% w/w CP lotion.

Fig. 5.2 to Fig. 5.4 compare the SC concentration versus depth profiles of vanillic acid, nepetin and hispidulin, respectively, from the two formulations after a 6-hour application. Vanillic acid penetrated the least and remained at a low concentration throughout the SC.

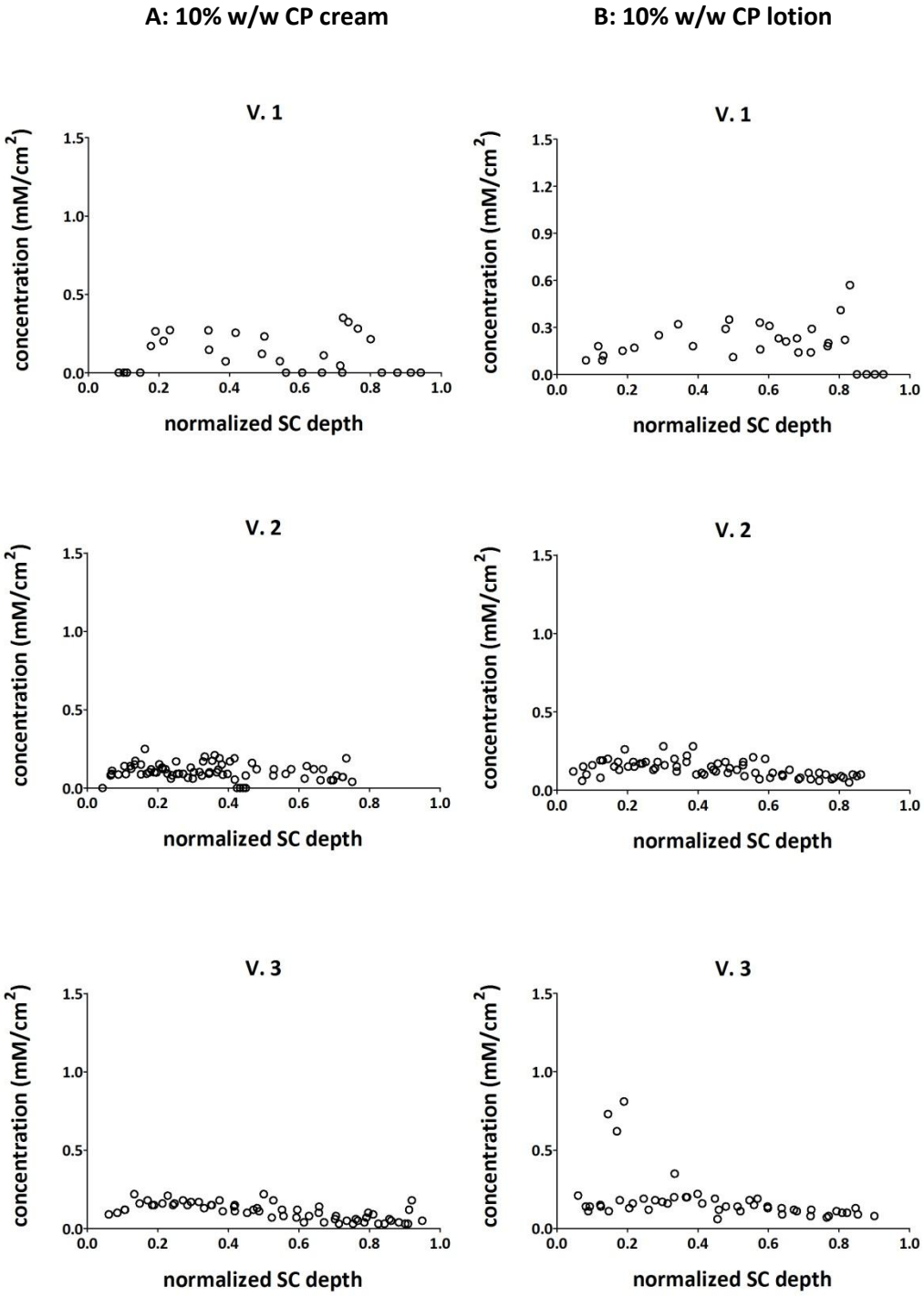


Fig. 5.2 SC concentration versus depth profiles of vanillic acid in subjects (V. 1-3, n = 3) after a 6-hour application of 10% w/w CP cream (panel A) and 10% w/w CP lotion (panel B):- continued overleaf.

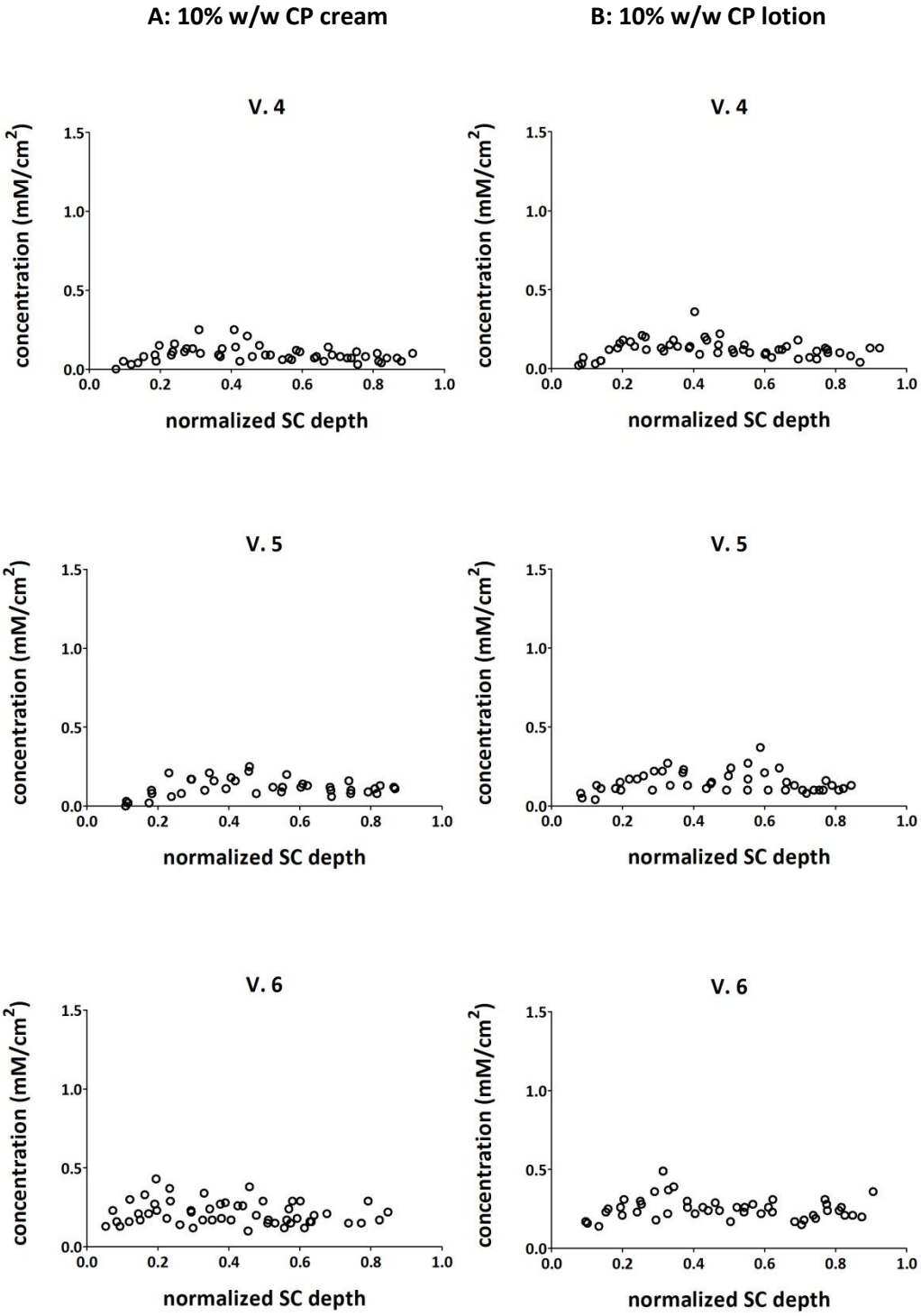


Fig. 5.2 cont. SC concentration versus depth profiles of vanillic acid in subjects (V. 4-6, n = 3) after a 6-hour application of 10% w/w CP cream (panel A) and 10% w/w CP lotion (panel B).

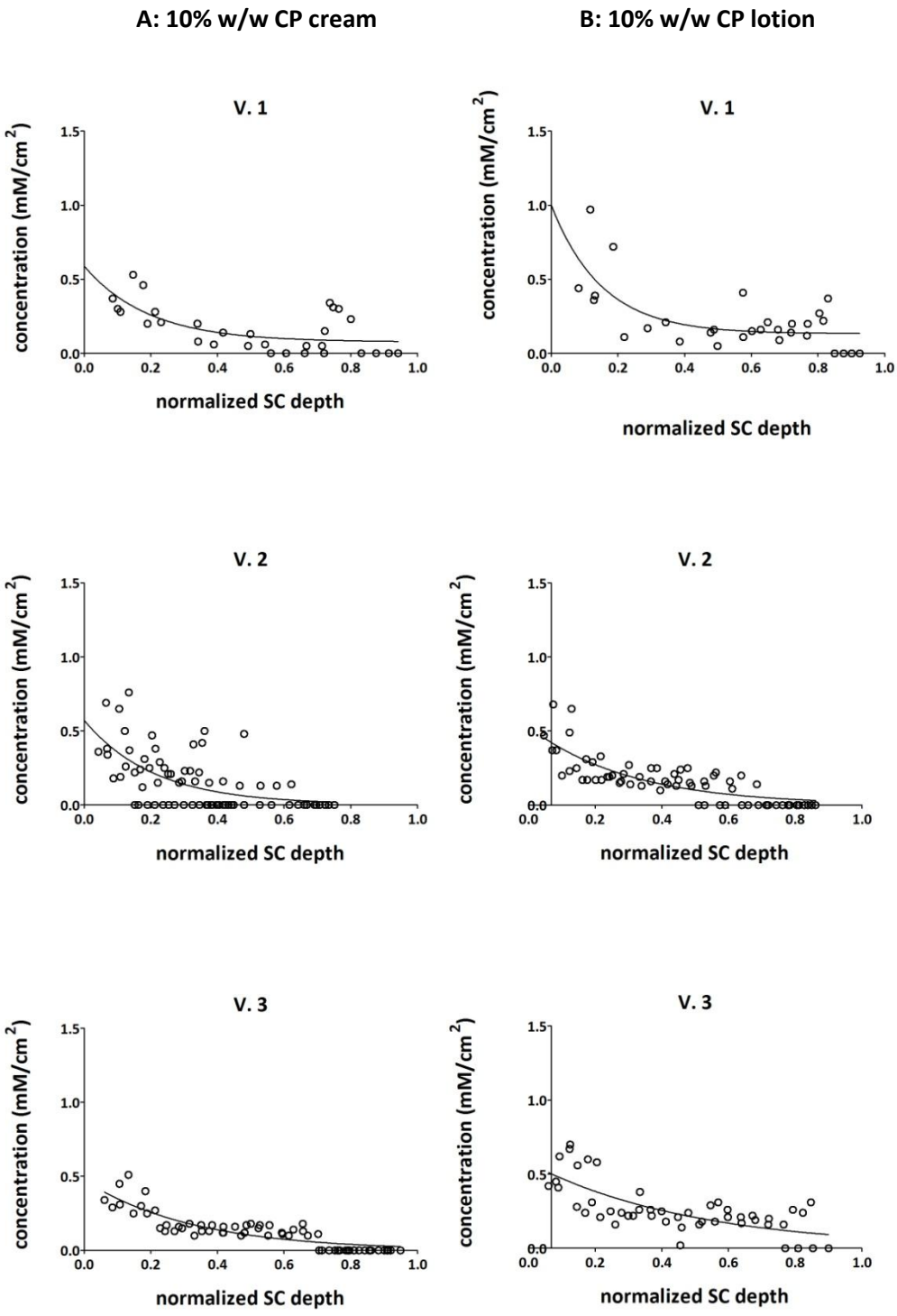


Fig. 5.3 SC concentration versus depth profiles of nepetin in subjects (V. 1-3, n = 3) after a 6-hour application of 10% w/w CP cream (panel A) and 10% w/w CP lotion (panel B):- continued overleaf.

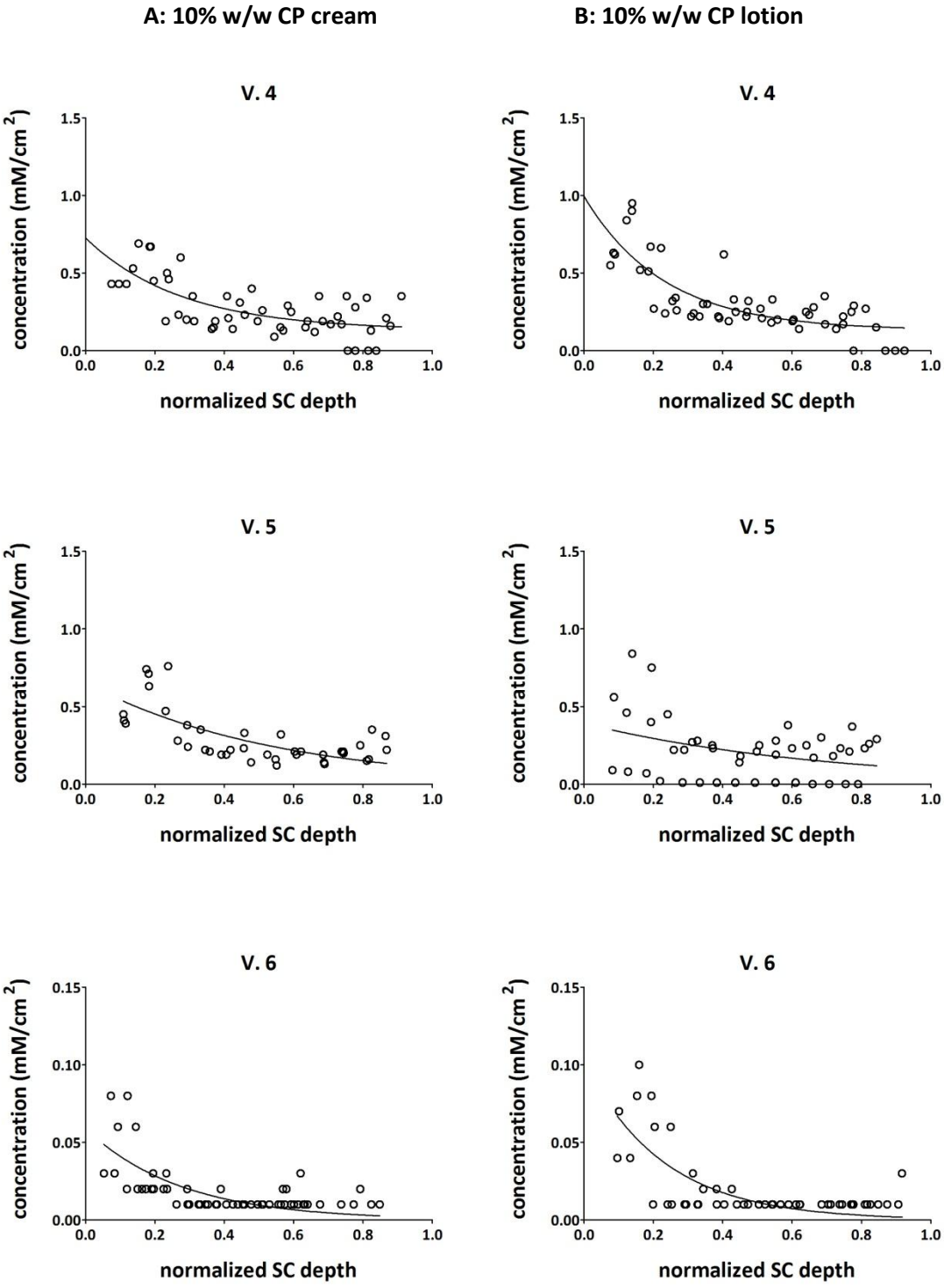


Fig. 5.3 cont. SC concentration versus depth profiles of nepetin in subjects (V. 4-6, n = 3) after a 6-hour application of 10% w/w CP cream (panel A) and 10% w/w CP lotion (panel B).

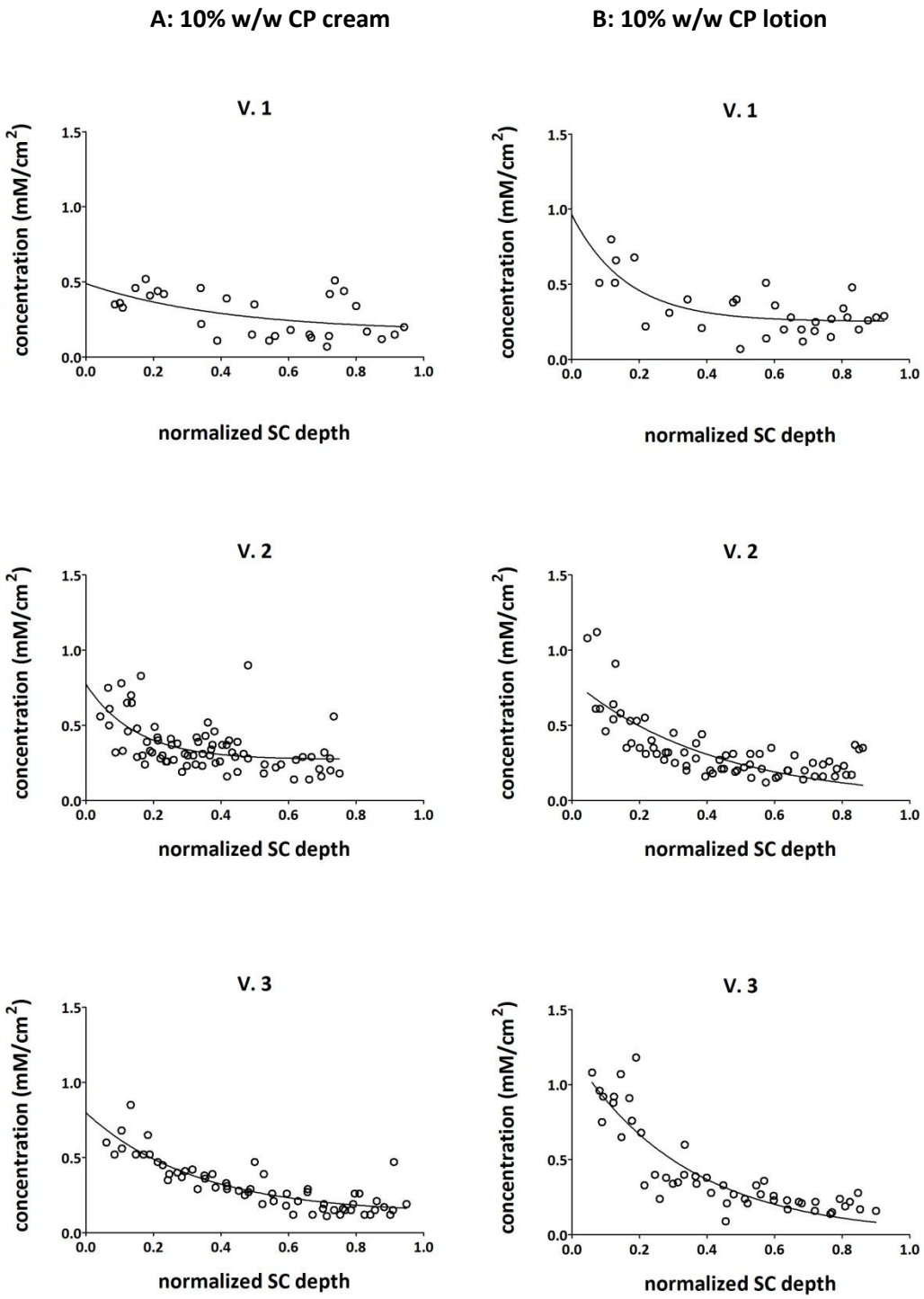


Fig. 5.4 SC concentration versus depth profiles of hispidulin in subjects (V. 1-3, n = 3) after a 6-hour application of 10% w/w CP cream (panel A) and 10% w/w CP lotion (panel B):- continued overleaf.

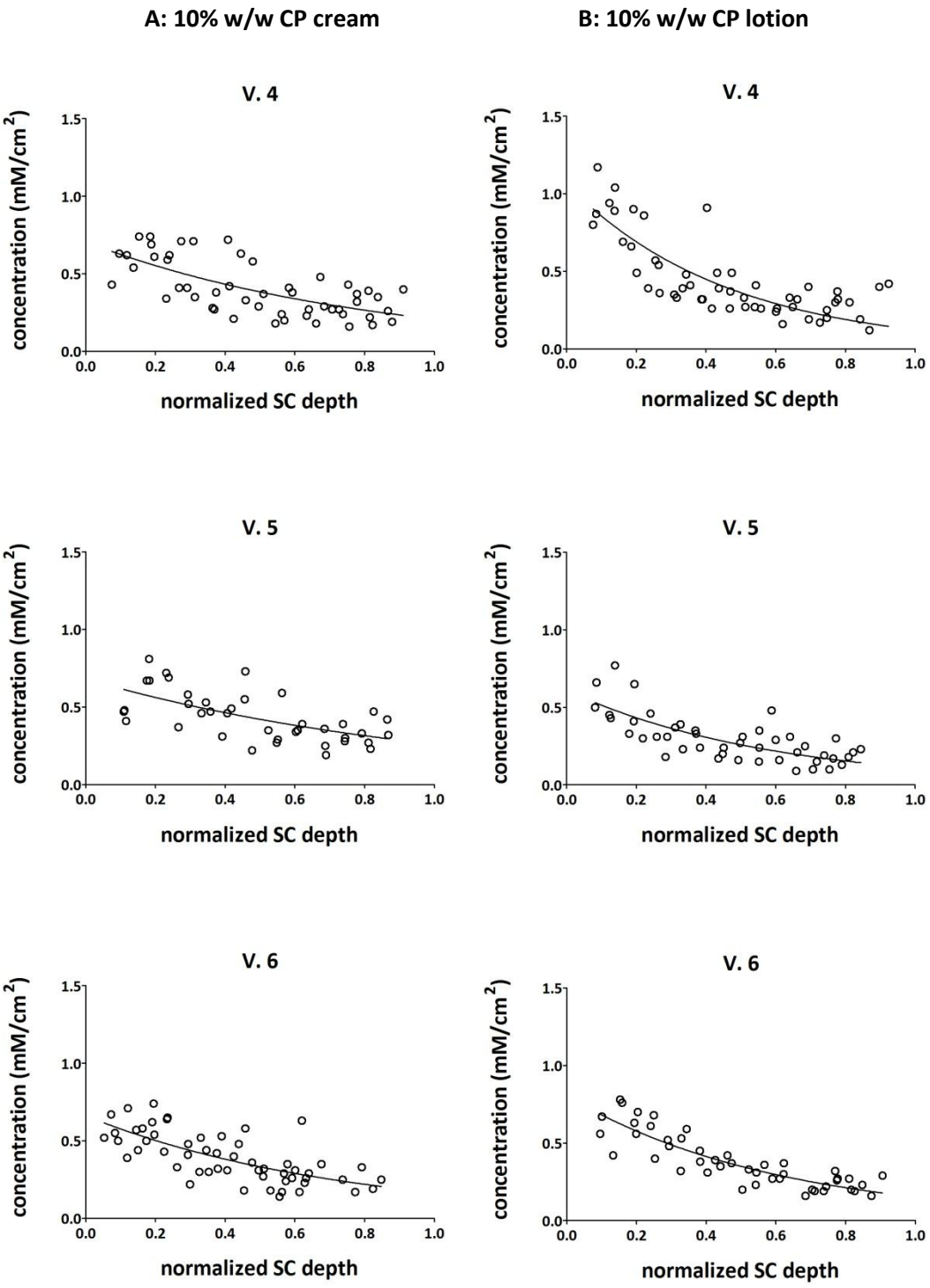


Fig. 5.4 cont. SC concentration versus depth profiles of hispidulin in subjects (V. 4-6, n = 3) after a 6-hour application of 10% w/w CP cream (panel A) and 10% w/w CP lotion (panel B).

Concentration-depth profiles of nepetin and hispdulin have been fitted to an infinite dose model (Alberti *et al.*, 2001; Herkenne *et al.*, 2007; Wagner *et al.*, 2000). From a visual inspection of the profiles, the apparent intercepts are all in the range 0.5-1.5. This implies that the SC-vehicle partition coefficients ($K_{SC,V}$) of nepetin is about 2.6 times that of hispidulin given that their concentrations in the formulations were 1.5 and 3.9 µmol/g, respectively. However, the result for nepetin in volunteer 6, when the MS assay was used, would point to a value of $K_{SC,V}$ which is approximately one-quarter of that for hispidulin.

The SC thickness at the skin sites treated with the control formulations (cream and lotion bases without the plant extracts) and the 10% w/w CP formulations was measured gravimetrically (Fig. 5.5). There was no significant difference in the values observed.

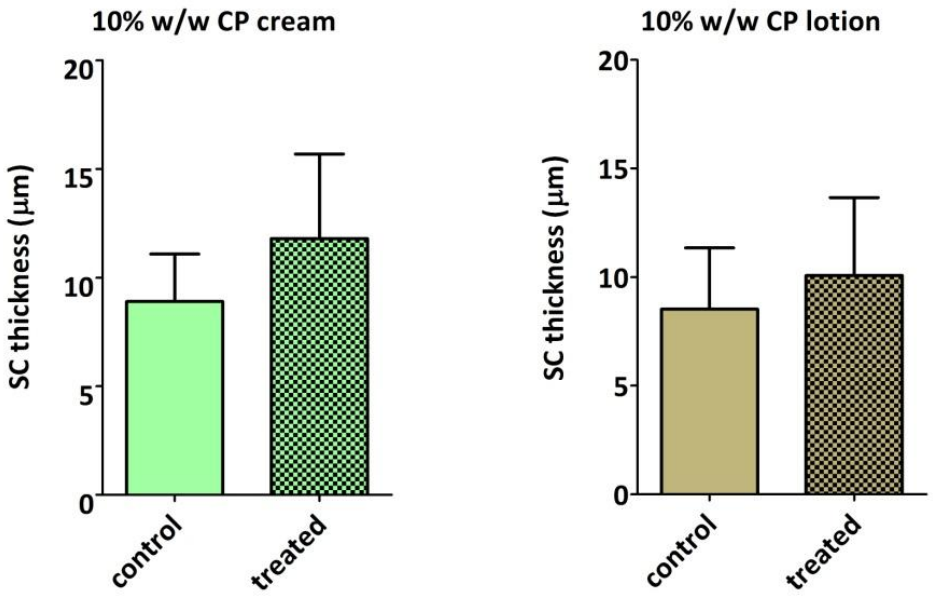


Fig. 5.5 SC thickness at skin sites treated with control formulations (n = 6), a 10% w/w CP cream and a 10% w/w CP lotion (n = 18).

The formulations themselves elicited no visible effect on the skin, in terms of irritation, for example. As is typically the case, tape-stripping the SC did cause visible redness at the skin site, and some dryness for a few days post-stripping.

5.2.3 *In vitro* and *in vivo* comparison

A comparison between the *in vivo* and *in vitro* data (from Chapter 4) is illustrated in Fig. 5.6. The graphs show the same pattern of behaviour for three compounds with verbascoside the exception. SC uptake *in vitro* was higher than *in vivo* and statistically significant differences were found for nepetin and hispidulin ($P < 0.01$ and < 0.001, respectively). Verbascoside was present in the SC below its LOQ *in vivo* as discussed before.

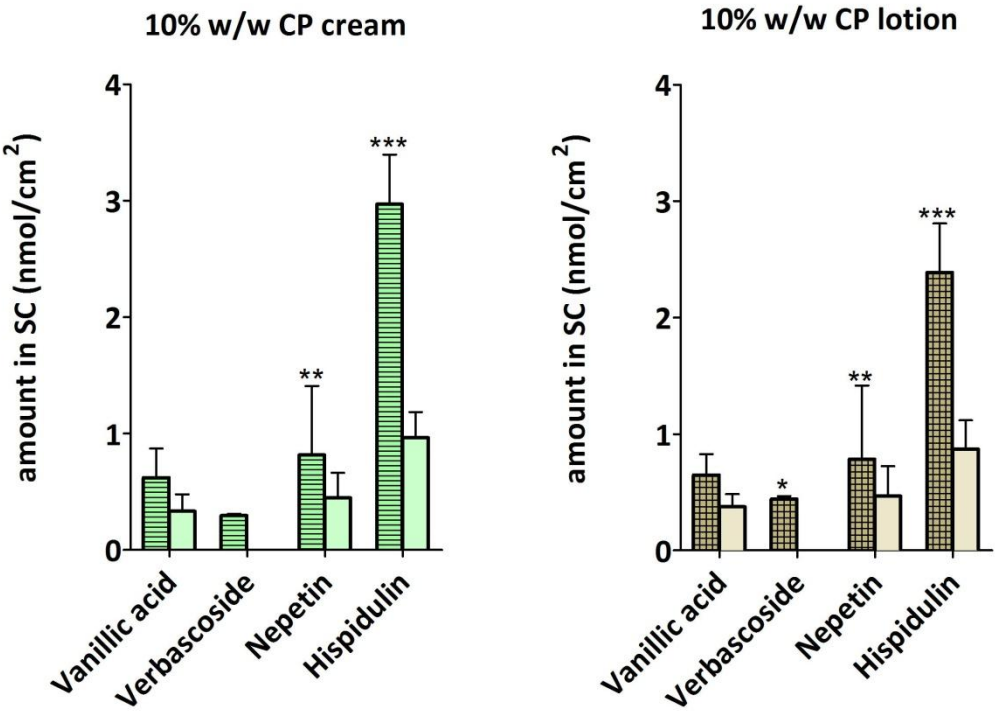


Fig. 5.6 Amounts (mean ± SD) of vanillic acid, verbascoside, nepetin and hispidulin taken up into the SC during the 6-hour *in vitro* and *in vivo* permeation studies (n=6). Striped green and solid green bars correspond to the *in vitro* and *in vivo* data from 10% w/w CP cream, respectively; hashed brown and solid brown bars correspond to the *in vitro* and *in vivo* data from 10% w/w CP lotion, respectively.

5.3 Conclusions

From the *in vivo* results, the presence of hispidulin, nepetin and vanillic acid in SC were quantified after a 6-hour application of both a cream and a lotion containing 10% w/w *C. petasites*. Hispidulin was taken up into the SC in greater amounts than vanillic acid and nepetin. Vanillic acid remained at low concentration throughout the SC whereas, the absorption of nepetin and hispidulin resulted in SC concentration profiles typically seen

after infinite dosing. Only verbascoside was unable to be quantified. No significant difference was observed between the two formulations.

A reasonable *in vitro-in vivo* correlation in the SC uptake data was observed, and the formulations tested were benign on the skin *in vivo*.

Chapter 6 *In vitro* release profiles of a cream and a lotion containing 10% w/w *C. petasites*

6.1 Study objective

For topical semisolid formulations, such as creams and lotions, *in vitro* release tests (IVRTs) using Franz diffusion cells are recommended by the Food and Drug Administration (FDA) as one method to assure product quality (Guidance for industry: Nonsterile semisolid dosage forms, 1997). Data from IVRTs can also be used to evaluate the impact of different vehicles or excipients on drug release from topical products and enable the performance between different formulations to be compared. Drug release rates are calculated from the linear slope of the plot of cumulative drug release versus the square root of time (Higuchi, 1961).

In the *in vitro* and *in vivo* skin penetration studies, there was no significant difference in the delivery of four target compounds between the 10% w/w CP cream and lotion. However, the excipient compositions of those formulations are not the same, potentially affecting the release properties of the active compounds in different ways.

The specific aims are:

- To determine release profiles of four phenolic markers, vanillic acid, verbascoside, nepetin and hispidulin from cream and lotion formulations containing 10% w/w *C. petasites*.
- To discriminate the release rates of the actives between the two topical preparations.
- To consider the present formulations in light of the results to optimise a suitable product for *C. petasites* in the future.

6.2 Results and discussion

6.2.1 Formulation analyses for *in vitro* release tests (IVRTs)

HPLC coupled with a UV detector was used to quantify the actives in the experimental formulation. Table 6.1 presents the assay performance characteristics for the compounds over the concentration range of 0.08-600 µM in acetonitrile. Limits of quantification (LOQs) of vanillic acid, nepetin and hispidulin were less than 2.4 µM; that of verbascoside was 32 µM.

Table 6.1 Retention time (t_R), selected wavelength (λ_{actual}), limit of quantification (LOQ; S/N ≥ 10); calibration curves (y = ax+b; r^2) of vanillic acid, verbascoside, nepetin and hispidulin standards (concentration range of 0.08-600 µM in acetonitrile, n = 3).

Compound	**t_R**	**λ_{actual}**	**LOQ**	**r^2**	**y = ax+b**	
	(min)	**(nm)**	**(µM)**		**a**	**b**
Vanillic acid	8.0	260	2.4	1.00	23780 ± 194	4822 ± 49070
Verbascoside	10.1	330	32.0	0.96	4643 ± 495	-180700 ± 53170
Nepetin	18.4	330	0.2	1.00	41470 ± 202	-65710 ± 23900
Hispidulin	19.5	330	0.2	1.00	50130 ± 41	-9020 ± 5116

Liquid-liquid partition with acetonitrile and hexane was used to extract the compounds from the topical formulations. The extraction procedure was validated by introducing 10 mL of a 5 $\mu g \cdot mL^{-1}$ standard mixture in acetonitrile and 30 mL hexane into 300 mg of cream/lotion base. This mixture was shaken vigorously and sonicated for 10 minutes before separating in a 50 mL glass funnel. The acetonitrile fraction was collected and filtered through 0.45 µm nylon membrane prior to UV detection.

Extraction efficiencies of the cream and lotion are in Table 6.2. Recovery of vanillic acid, nepetin and hispidulin was acceptable (80-93%); however, verbascoside was not quantifiable as the amount present was below its LOQ, but it was detectable.

Table 6.2 Validation of cream and lotion extraction (n = 3) after spiking with a standard mixture of vanillic acid, verbascoside, nepetin and hispidulin (5 µg·mL^{-1} in acetonitrile).

Formulation	Compound	Theoretical concentration (µM)	Actual concentration (average, µM)	SD	%RSD	% Recovery
Cream	Vanillic acid	29.5	27.4	1.3	4.7	92.9
	Verbascoside	7.5	-	-	-	-
	Nepetin	15.9	12.4	0.1	0.8	78.3
	Hispidulin	16.7	15.5	0.1	0.7	93.2
Lotion	Vanillic acid	29.5	27.4	0.4	1.3	92.9
	Verbascoside	7.5	-	-	-	-
	Nepetin	15.9	13.4	0.01	0.1	84.3
	Hispidulin	16.7	15.5	0.03	0.2	93.1

Three replicates each of 300 and 600 mg cream/lotion samples containing 10% w/w *C. petasites* were extracted and the four compounds quantified (Table 6.3). The results were very reproducible confirming the consistent formulation preparation process. Apart from verbascoside, the levels of each compound were similar to those predicted from their HPLC-MS quantification (Table 3.10, Chapter 3).

Table 6.3 Quantities of vanillic acid, verbascoside, nepetin, and hispidulin measured in cream and lotion formulations containing 10% w/w *C. petasites* following the extraction procedure with acetonitrile and hexane (n = 6; 300 and 600 mg formulation samples).

	Amount (µmol/g, average ± SD) in the formulation			
Formulation	**Vanillic acid**	**Verbascoside**	**Nepetin**	**Hispidulin**
10% w/w CP cream	0.2 ± 0.01	1.0 ± 0.2	1.2 ± 0.1	3.9 ± 0.2
10% w/w CP lotion	0.2 ± 0.01	1.1 ± 0.2	1.3 ± 0.04	4.0 ± 0.2
10% w/w CP cream/lotion[a]	0.3	0.4	1.5	3.9

a = values calculated from quantification of the extract by direct injection (Table 3.10, Chapter 3)

6.2.2 Sink condition and back diffusion

In all IVRTs, the receptor medium must provide sink conditions in which the concentration of the compounds of interest never exceeds 10% of their solubility throughout the course of the experiment (Topical and transdermal drug products, 2009). In this study, therefore, 10% of the compounds' saturation solubilities in the receptor solution (a 1:4 v/v mixture of ethanol and 5 mM Tris buffer) was the criterion used to ensure adequate sink conditions (Table 6.4). The receptor medium was also replaced at any sampling time when its colour had become tinged with dark green from the plant product formulations.

Table 6.4 Saturation solubilities of vanillic acid, verbascoside, nepetin, and hispidulin in the receptor solution (a 1:4 v/v mixture of ethanol and 5 mM Tris buffer).

Compound	Solubility (mM)
Vanillic acid	34.6
Verbascoside	7.5
Nepetin	0.5
Hispidulin	0.1

Back diffusion of the compounds of interest from the receptor compartment to the donor chamber during the experiment must also be avoided and requires that the penetrant's level in the receptor solution is less than 30% of that in the applied formulation (Marangon *et al.*, 2009). In all IVRTs performed here, this condition was fulfilled.

6.2.3 Membrane selection and experimental period

IVRTs aim to study the release properties of compounds from formulations. Therefore, the membrane used should not hinder penetration to the receptor compartment; it should simply provide an inert physical barrier separating the receptor solution from the formulation. Artificial membranes are extensively used in IVRTs and there are many examples available commercially.

In this study, Tuffryn® (a polysulfone membrane; 145 µm thick with 0.45 µm pore size) and Spectra/Por® (a dialysis membrane; MWCO: 1000) membranes were examined for their suitability for further investigation. Pre-treatment of membranes with the receiving medium is recommended to replace air within the pores by the liquid (Zatz and Segers, 1998). Spectra/Por® membranes are already pre-soaked in 0.05% sodium azide (aq.). Hence, only the Tuffryn® membranes were treated with the receptor solution (a 1:4 v/v mixture of ethanol and 5 mM Tris buffer). Isopropyl myristate (IPM) was also used here as a membrane wetting agent because it has been suggested as a useful model of skin lipids (Poulsen *et al.*, 1968). This allowed the IVRT results, in this case, to be compared with the penetration results from pig skin.

Pilot experiments with the 10% w/w CP lotion were performed over 72 hours using three different membranes: Tuffryn® membrane soaked with IPM (M1), Tuffryn® membrane soaked with the receptor solution (a 1:4 v/v mixture of ethanol and 5 mM Tris buffer) (M2), and Spectra/Por® membrane (M3). Sampling times were 0.25, 0.5, 0.75, 1, 1.5, 2, 3, 4, 5, 6,

8, 24, 32, 48, 56, and 72 hours. Release rate profiles of vanillic acid, verbascoside, nepetin and hispidulin are presented in Fig. 6.1.

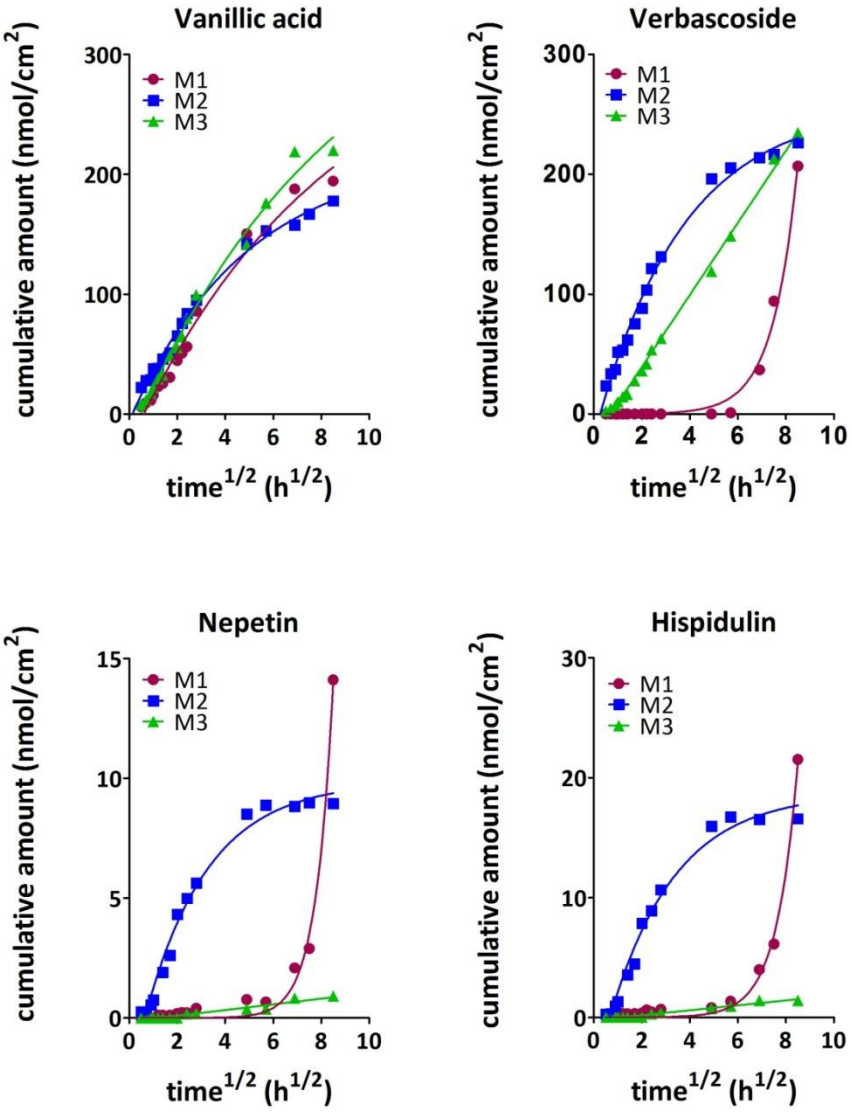


Fig. 6.1 *In vitro* release profiles of four compounds from a 10% w/w CP lotion through different membranes over 72 hours. M1: Tuffryn® membrane soaked with isopropyl myristate (IPM); M2: Tuffryn® membrane soaked with the receptor solution (a 1:4 v/v mixture of ethanol and 5 mM Tris buffer); M3: Spectra/Por® membrane.

Release of vanillic acid across the three membranes was similar and relatively rapid. The release of verbascoside, nepetin and hispidulin was greatest across M2. In contrast, the transfer of nepetin and hispidulin across M3 was very slow, suggesting the unsuitability of this membrane for a simple IVRT. The precise reason for this finding could not be unambiguously determined from this preliminary experiment.

Apart from vanillic acid, the IPM-treated membrane (M1) acted as an obvious diffusional barrier to the other compounds, with clear lag-times being apparent. Similar to the *in vitro* skin results, detectable amounts of nepetin and hispidulin had crossed M1 by 3 hours post-application. The measurable transport of verbascoside observed at 32 hours calls into question the retention efficiency of IPM in M1. It is possible that the ethanol in the receptor phase may progressively dissolve out the IPM within the membrane over time, and that the rapid increase in transport then observed may be an experimental artifact.

Overall, the Tuffryn® membrane soaked with the receiving medium (M2) offered the least barrier to all compounds and the most appropriate choice for the IVRT experiments. The 8-hour release data for vanillic acid, verbascoside, nepetin and hispidulin were linear with the square root of time and are re-plotted in Fig. 6.2. The corresponding release rates are in Table 6.5.

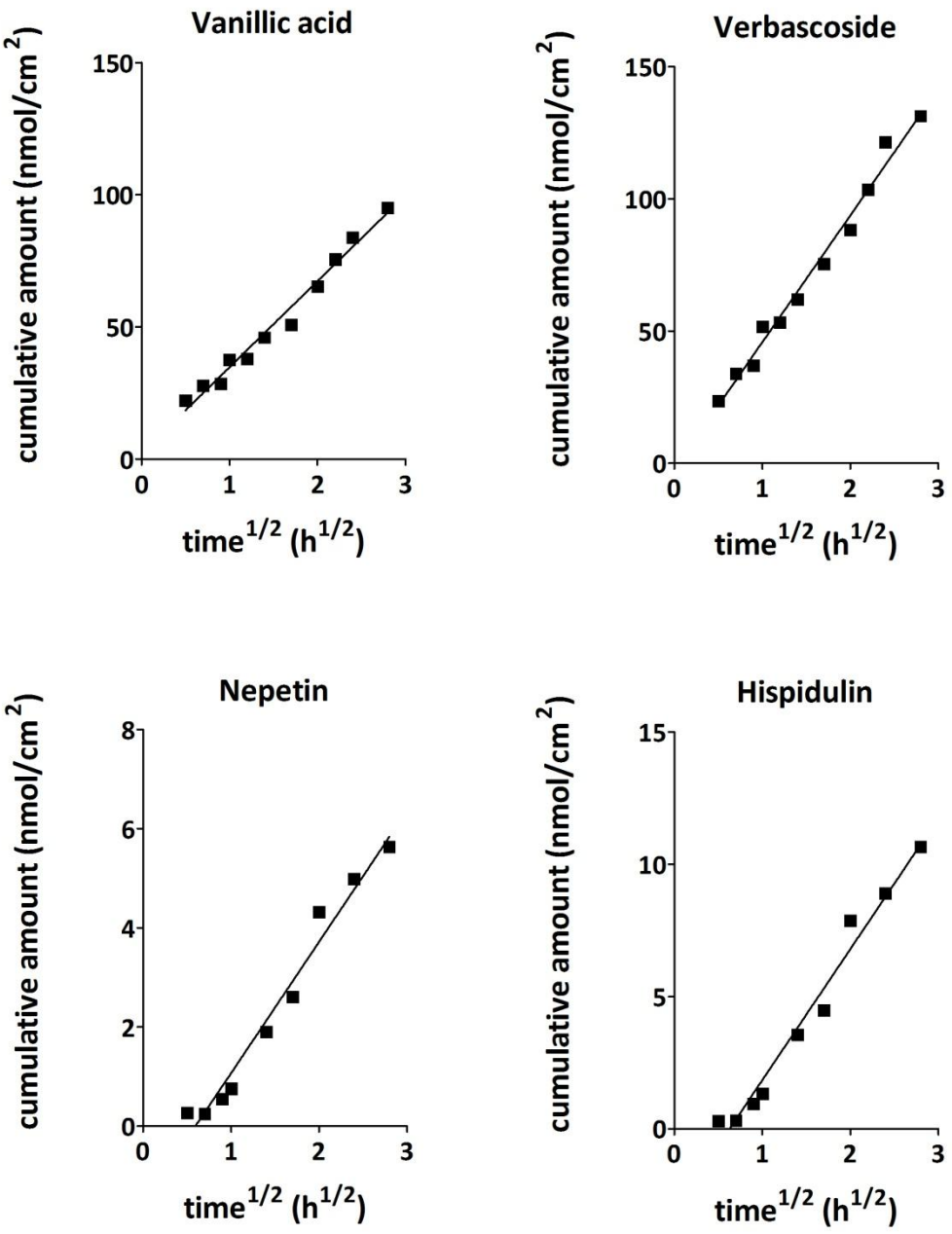


Fig. 6.2 *In vitro* release profiles over 8 hours of four compounds from 10% w/w CP lotion across a Tuffryn® membrane soaked with the receptor solution (a 1:4 v/v mixture of ethanol and 5 mM Tris buffer).

Table 6.5 Release rates of vanillic acid, verbascoside, nepetin and hispidulin from a 10% w/w CP across a Tuffryn® membrane soaked with the receptor solution (a 1:4 v/v mixture of ethanol and 5 mM Tris buffer).

Compound	M2 Tuffryn® membrane (soaked with receptor solution)	
	Flux ($nmol \cdot cm^{-2} \cdot h^{-1/2}$)	r^2
Vanillic acid	32.5	0.98
Verbascoside	47.9	0.99
Nepetin	2.7	0.97
Hispidulin	5.0	0.97

6.2.4 Membrane-binding study

Next, IVRTs of three different dosage forms, a cream, a lotion and a solution were performed. The amount of the plant extract formulated in the products was controlled at 10% w/w. The results are in Fig. 6.3.

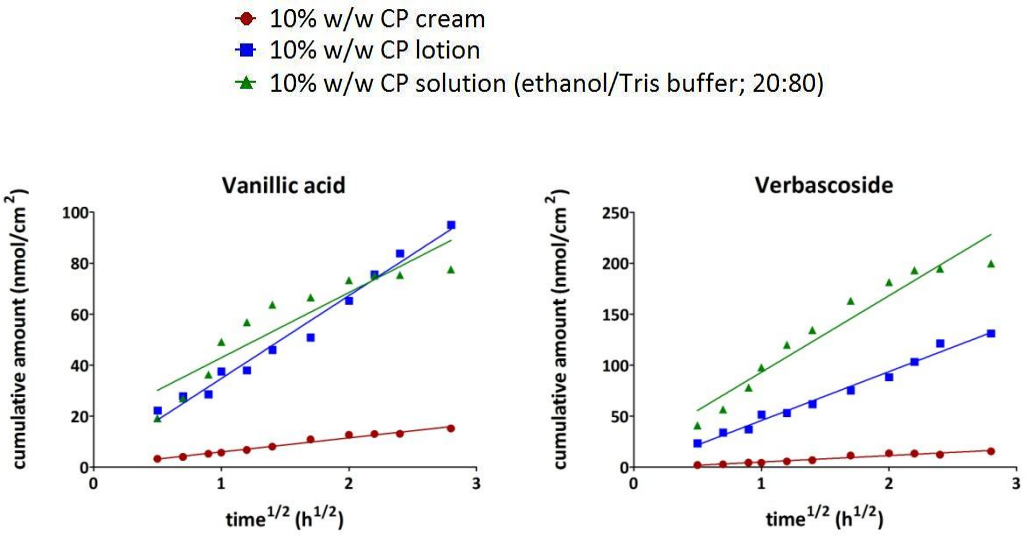


Fig. 6.3 *In vitro* release over 8 hours of vanillic acid and verbascoside from three formulations across a Tuffryn® membrane soaked with the receptor solution.

It is clear that the release of verbascoside from a CP solution was fastest. For vanillic acid, release from the cream was obviously slower than that from either the solution or the lotion. Even over 8 hours, it appears that some depletion of these two compounds is occurring and the release rates are beginning to tail off (especially for the solution, where

loss of ethanol from the donor may also have an effect). Nepetin and hispidulin were only detected in trace amounts in these experiments and could not be quantified.

6.2.5 Release rate profiles from cream and lotion formulations containing 10% w/w *C. petasites*

Three IVRT replicates over 8 hours using Tuffyn® membranes pre-treated with the receiving medium of each topical product (10% w/w CP cream and lotion) were performed. Release profiles of vanillic acid and verbascoside from the cream are in Fig. 6.4, with the percentage release and release rates are in Table 6.6.

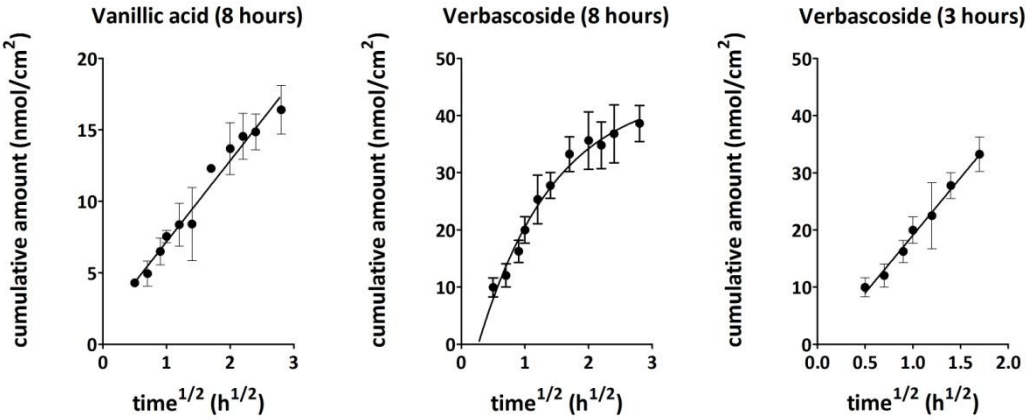


Fig. 6.4 Release profiles of vanillic acid and verbascoside from a 10% w/w CP cream over 8 hours (n = 3).

The release rate of verbascoside was faster than vanillic acid but by a factor ~3.5 less than the difference in their concentration in the cream (~6-fold). The release profile of verbascoside was not completely linear over 8 hours. This would not be due to the depletion of the compound in the cream since less than 7% had been released over this period. To compare release rates between vanillic acid and verbascoside, therefore, it was decided to use only data up to 3 hours for the latter compound. Again, nepetin and hispidulin were detected only in trace amounts which may presumably be explained by their relatively higher affinity towards the formulations.

Table 6.6 Quantity, percentage release and release rate of vanillic acid, verbascoside, nepetin and hispidulin from a 10% w/w CP cream over 8 hours (n = 3).

Compound	Quantity ($nmol \cdot cm^{-2}$)		% release	Release rate ($nmol \cdot cm^{-2} \cdot h^{-1/2}$)	r^2
	Reservoir[a]	Receptor[b,c]			
Vanillic acid	94 ± 2.4	16.4 ± 1.7	17.5 ± 1.9	5.7 ± 0.3	0.91
Verbascoside	584 ± 15	38.6 ± 3.2	6.6 ± 0.6	20.2 ± 1.6[d]	0.90[d]
Nepetin	706 ± 18	trace	-	-	-
Hispidulin	2,242 ± 57	trace	-	-	-

a = UV detection; b = MS detection; c = freeze dry; d = linear over 3 hours

IVRT of the 10% CP lotion yielded the results in Fig. 6.5 and Table 6.7. Again, nepetin and hispidulin were not quantified. Despite the presence of vanillic acid and verbascoside in similar amounts to those present in the cream, their release rates (and % release) were significantly higher. Indeed, over 90% of the vanillic acid originally applied to the membrane was released in 8 hours. However, intriguingly, the release rate remained constant and did not show any sign of tailing off (as would normally be anticipated); the reason for this anomaly is not known.

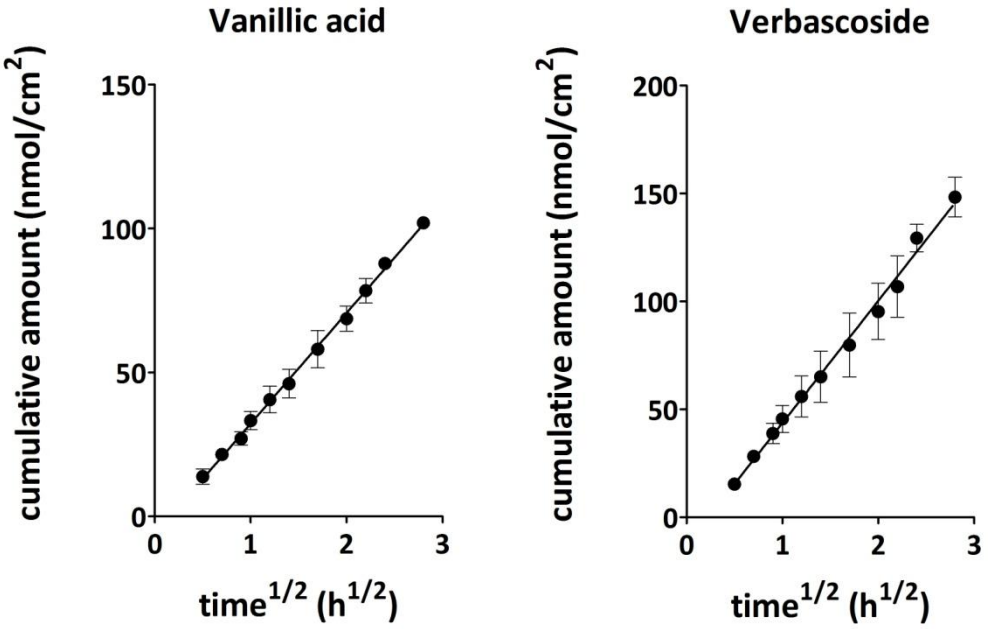


Fig. 6.5 Release profiles of vanillic acid and verbascoside from a 10% w/w CP lotion over 8 hours (n = 3).

Table 6.7 Quantity, percentage release and release rate of vanillic acid, verbascoside, nepetin and hispidulin from a 10% w/w CP lotion over 8 hours (n = 3).

Compound	Quantity ($nmol \cdot cm^{-2}$)		% release	Release rate ($nmol \cdot cm^{-2} \cdot h^{-1/2}$)	r^2
	Reservoir[a]	Receptor[b]			
Vanillic acid	113 ± 1	102 ± 1	90.1 ± 0.8	38.4 ± 0.8	0.99
Verbascoside	631 ± 3	148 ± 9	23.5 ± 1.4	56.6 ± 2.1	0.96
Nepetin	750 ± 3	trace	-	-	-
Hispidulin	2,261 ± 9	trace	-	-	-

a = UV detection; b = MS detection

6.3 Conclusions

In vitro release tests (IVRTs), using polysulfone membranes (Tuffryn®) pre-soaked with a 1:4 v/v mixture of ethanol and 5mM Tris buffer as a receptor solution, enabled 8-hour release profiles of vanillic acid and verbascoside from cream and lotion formulations containing 10% w/w *C. petasites* to be determined. Unfortunately, the method was not suitable for nepetin and hispidulin, for which a different receiving medium and/or membrane may be required.

The release of vanillic acid and verbascoside from the lotion was 4-5 times higher over 8 hours than that from the cream; however, these formulations showed no difference in their ability to deliver these compounds across the mammalian skin, either *in vitro* or *in vivo*. This confirms current wisdom that IVRT measurements are useful tests with which to compare formulations in development, and for the purposes of quality control, but they provide no quantitative prediction of performance in the clinic.

Chapter 7 Potential biological activities of topical cream and lotion formulations containing 10% w/w *C. petasites*

7.1 Purpose

In Thai traditional medicines, *C. petasites* is used to treat many skin problems, such as rash, abscess, urticaria, snakebites and insect bites (Pongboonrot, 1965; Thai traditional medical textbook: Paet-Ta-Ya-Saat-Song-Kror (แพทยศาสตร์สงเคราะห์), 2007) (T. Tipcharoentham, pers. comm., 2011; S. Tungjitjaruen, pers. comm., 2011). However, there is limited scientific information concerning the biological activities of this plant. A few evaluations have been reported: an anti-inflammatory effect (Panthong *et al.*, 2003), a weak radical scavenging activity, and a moderate anti-mutagenicity (Singharachai *et al.*, 2011b).

In this thesis, four phenolic compounds, vanillic acid, verbascoside, nepetin and hispidulin, in *C. petasites* have been studied with respect to their skin permeation from different formulations but biological tests have not been conducted. However, our results from the *in vitro* and *in vivo* permeation studies, with the biological activities of these compounds reported elsewhere may be used to predict the potential bioactivities of the products used in Thai traditional medicine. This exercise may then inform the design of future biological studies on these formulations.

7.2 Discussion

Generally speaking, in dermatology, three treatment regions; surface, stratum corneum, viable epidermis and dermis, are of interest (Fig. 7.1). Each layer of the skin is targeted for different treatments. For example, antibiotics and antiseptics aim to attack microorganisms (bacteria and fungi) on the skin surface without requiring penetration through the stratum corneum (SC). In the SC, the treatment aims include increasing water content to improve pliability and accelerating desquamation to remove dead cells; these effects can be produced by emollients (moisturising agents) and exfoliants. Anti-inflammatory,

antipruritic, antihistamine, and antioxidant drugs have to be percutaneously absorbed to the viable epidermis and dermis to be effective.

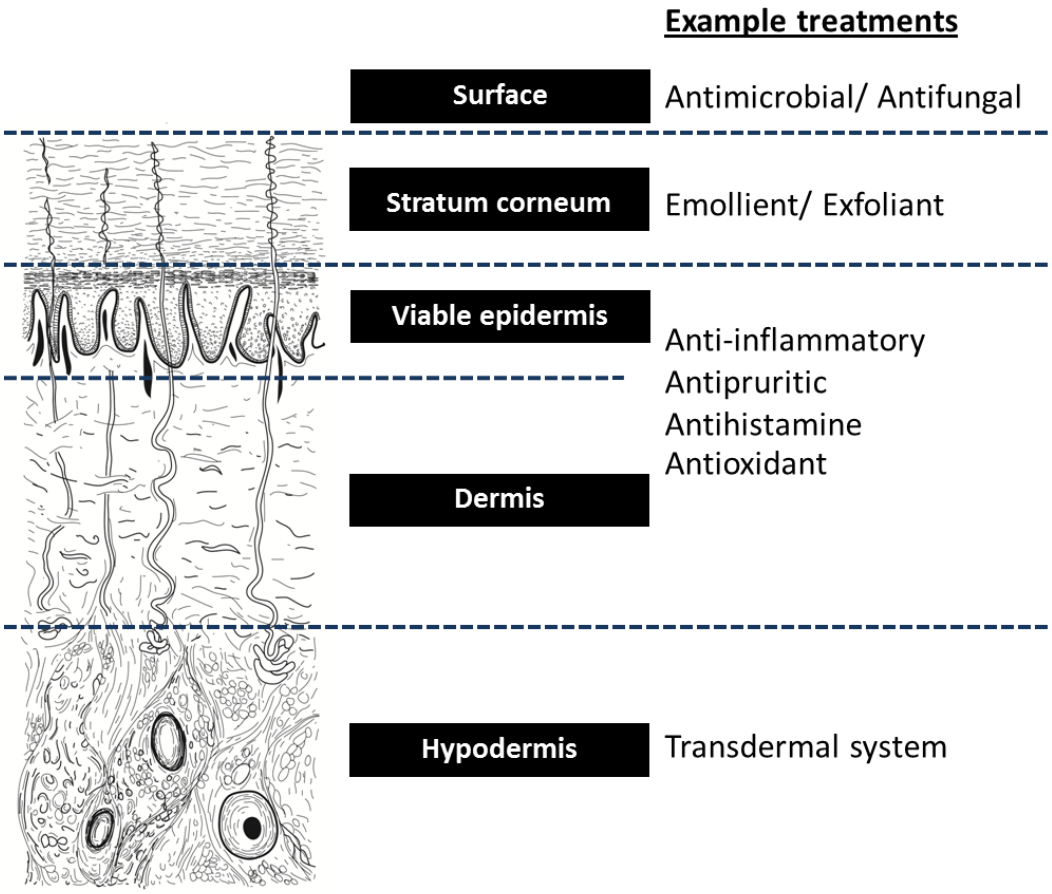


Fig. 7.1 Treatment concept for topical drug delivery systems (Barry, 1988). Note that transdermal drug delivery for systemic effect is not the subject of this thesis and is not discussed, therefore.

The biological activities of the naturally-occurring compounds in the CP cream and lotion may be considered in terms of potential effective doses and with respect to the chemical structures of the putative actives present in the plant.

7.2.1 Comparison of skin concentrations achieved experimentally with biologically effective doses reported in the literature

Concentrations of vanillic acid, verbascoside, nepetin and hispidulin present in the SC and achieved in the receptor solution in the *in vitro* permeation studies (Chapter 4), as well as their concentrations in the SC observed in the *in vivo* percutaneous absorption studies (Chapter 5), are displayed in Table 7.1 for the 10% w/w CP cream and lotion, respectively.

Table 7.1 Concentrations of vanillic acid, verbascoside, nepetin and hispidulin in the stratum corneum (*in vitro* and *in vivo*) and in the receptor solution (*in vitro*) following application of the 10% w/w CP cream and lotion.

Compound	**Vehicle**	**Average concentration (µM)**		
		SC (*in vitro*)[a]	**Receptor**[a]	**SC (*in vivo*)**[b]
Vanillic acid	Cream	389	0.15	290
	Lotion	499	0.10	395
Verbascoside	Cream	183	-	-
	Lotion	343	-	-
Nepetin	Cream	510	0.02	427
	Lotion	605	0.02	477
Hispidulin	Cream	1858	0.25	860
	Lotion	1838	0.17	894

[a] n = 6 (Chapter 4); [b] n = 18 (Chapter 5)

The receptor solution concentrations measured *in vitro* may be considered to reflect, at least relatively, an idea of the maximal values achievable in the viable skin layers. Given that skin *in vitro* lacks an active microcirculation and a mechanism with which to "clear" permeated drug, it is reasonable and logical to expect that the concentrations deduced from an *in vitro* experiment are over-estimates of those likely to be attained *in vivo*. Nevertheless, the similarities in the SC recoveries from the *in vitro* and *in vivo* studies are encouraging confirmations that the relative viable skin concentrations *in vivo* will parallel those found in the *in vitro* receptor solution.

7.2.1.1 Antimicrobial activity

Vanillic acid possesses antimicrobial propertiies (Delaquis *et al.*, 2005) against *Listeria monocytogenes*, *Listeria innocua*, *Listeria grayi* and *Listeria seeligeri* with an MIC of 10 mM at pH 5.

Antibacterial activity has been observed for nepetin (Sultana and Afolayan, 2007), which is active against *Bacillus subtilis*, *Staphylococcus aureus* and *Staphylococcus sonnei* with MICs of 4, 31, and 250 μg·mL^{-1} (equivalent to 13, 98, and 790 μM), respectively.

Shikanga *et al.* (2010) reported that verbascoside has antibacterial activity against *Straphylococcus aureus*, *Enterococcus faecalis*, *Escherichia coli*, and *Pseudomonas aeruginosa* with MICs of 0.06, 0.1, 0.1, and 0.25 mg·mL^{-1} (equivalent to 0.1, 0.2, 0.2, and 0.4 mM), respectively.

7.2.1.2 Anti-inflammatory activity

Kim *et al.* (2011) reported that vanillic acid, at a concentration of ≥ 10 μM, has an anti-inflammatory effect via inhibition of lipopolysaccharide (LPS)-induced secretion of tumour necrosis factor (TNF)-α and interleukin (IL)-6; it also decreases the levels of cyclooxygenase (COX)-2 and nitric oxide (NO) during the inflammatory process in mouse peritoneal macrophages. Interestingly, vanillic acid (≥ 100 μM) also suppressed the activation of nuclear factor-kappa B (NF-κB) and capase-1 which play important roles in inflammatory responses, gene transcription and apoptosis.

Nepetin at a concentration of 40 μM possesses anti-inflammatory activity and shows 91% inhibition of NF-κB induction (Clavin *et al.*, 2007). It also reduced tetradecanoylphorbol acetate (TPA)-induced mouse ear oedema by 50% (1 mg/rat ear).

Hispidulin isolated from *Clerodendrum indicum* L reduced TPA-induced mouse ear oedema with an ED_{50} of 223 μg/mouse ear (Gil *et al.*, 1994). It is noted that the hispidulin used in this study was isolated from a plant in the same genus as *C. petasites*.

7.2.1.3 Antioxidant

The peroxynitrite ($ONOO^-$) scavenging activity of vanillic acid was investigated by Kang *et al.* (2009) and the IC_{50} was 7.6 μM.

Based on this information, together with the results in Table 7.1, it seems possible that the optimised cream and lotion formulations containing 10% w/w *C. petasites* may have antimicrobial activity. The sub-SC concentration of nepetin achieved from the formulations is higher than the effective concentrations reported for this activity.

Verbascoside could not be quantified in the SC *in vivo* but was detectable *in vitro* (183 and 343 µM for cream and lotion, respectively, Table 7.1). Potentially, therefore, verbascoside may be able to inhibit bacteria on the skin surface even though the compound does not penetrate through the skin. The only prerequisite for this is that verbascoside is released from the formulation, a fact confirmed in the *in vitro* release test in Chapter 6.

7.2.2 Potential bioactivities deduced from chemical structures

7.2.2.1 Flavonoids

The basic flavonoid structure, with the ring numbering system, and the chemical structures of the seven naturally-occurring flavonoids (chrysin, apigenin, luteolin, nepetin, hispidulin, naringenin and hesperetin) in *C. petasites* are illustrated in Fig. 7.2. Flavones comprise two aromatic (A and B rings) and one heterocyclic ring (C ring), a 2,3-double bond and a 4-keto group. The double bond is absent in flavanones (e.g., naringenin and hesperetin).

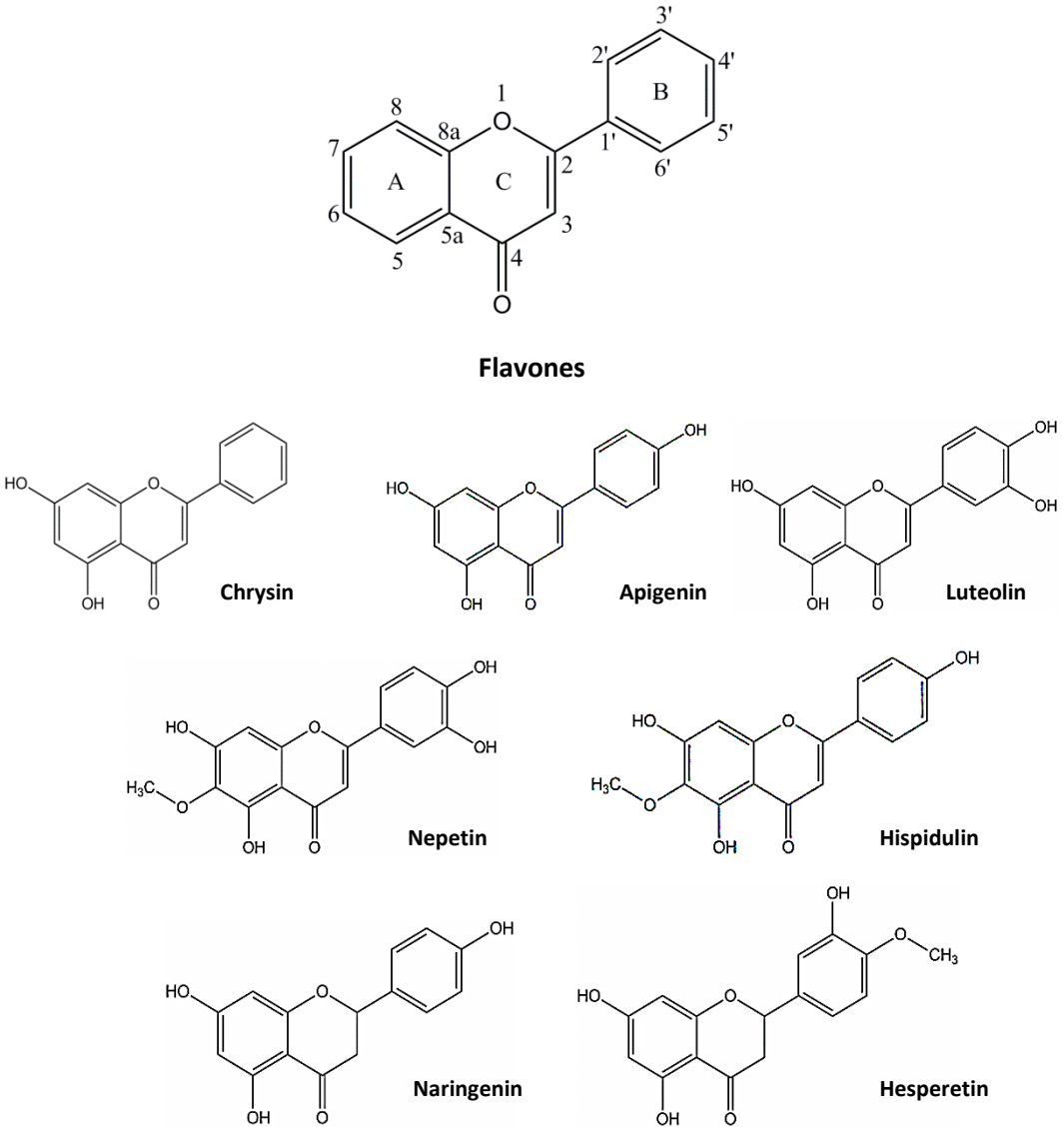


Fig. 7.2 Basic structure and numbering system of flavones and chemical structures of naturally-occurring flavonoids in *C. petasites*.

7.2.2.1.1 Antimicrobial activity

Generally, hydroxyl groups in flavanones and flavones are required for antimicrobial activity against methicillin-resistant *Straphylococcus aureus* (MRSA) (Alcaraz *et al.*, 2000). However, single 3'-OH and 4'-OH substitution showed less activity against the organism (Liu *et al.*, 2010). 2',4'- or 2',6'-dihydroxylation in the B ring and 5,7-dihydroxylation in the A ring of flavanones are important for anti-MRSA activity (Tsuchiya *et al.*, 1996); on the other hand, substitution of methoxyl groups drastically decreases the activity (Alcaraz *et al.*, 2000).

Glycosylation has also been suggested to suppress antimicrobial activity; therefore, aglycones are more active than glycosides (Liu *et al.*, 2010). Moreover, the attachment of hydrophilic substituents to the 7-*O*-aglycone and acylation at C3 in the C ring can reduce potency (Celiz *et al.*, 2011).

Modification of flavonoid structures by alkylation was reported to enhance efficacy. Molecules with relatively longer aliphatic chains at C6 or C8 are more active. However, attachment of a saturated aliphatic chain with 10-12 carbon atoms in the A ring (or to sugars attached to this ring by esterification) appears to result in the most promising structure to inhibit gram positive bacteria, such as *Listeria monocytogenes*, *Escherichia coli*, and *S. aureus* (Celiz *et al.*, 2011). Flavonoids with a 2-carbon spacer at C7 also show increased inhibition of both gram positive (e.g., *Straphylococcus subtilis*, *S. aureus*) and gram negative bacteria (e.g., *E. coli*, and *Psudomonas flurescens*) (Lv *et al.*, 2009).

Overall, all seven flavonoids identified in this study have the potential for antimicrobial and anti-MRSA activity. The relative potency is predicted to be chrysin > hispidulin (4'-OH) ≥ apigenin (4'OH) ≥ naringenin (4'-OH) > nepetin (3'- and 4'-OH) ≥ luteolin (3'- and 4'-OH) > hesperetin (3'-OH and 4'-OCH_3).

7.2.2.1.2 Anti-inflammatory activity

Gerritsen *et al.* (1995) suggested that flavonoids should have a 4-keto group and a 2,3-double bond to inhibit intercellular adhesion molecule (ICAM)-1 expression in the inflammatory cascade. The substitution of 3'-OCH_3 or 3'-OH slightly reduces potency while the presence of 3-OH strongly reduces the inhibitory activity. Replacement of hydroxyl group at C5 and C7 with a methoxyl group or a sugar completely destroys efficacy. Furthermore, the B ring must be positioned off C2 and not C3; otherwise, the inhibitory activity is massively reduced.

A structure-bioactivity relationship for the inhibition of LPS-stimulated TNF-α release was reported by Xagorari *et al.* (2001). To show this activity, flavonoids must have the B ring positioned off C2 together with a 2,3-

double bond and 4-keto group. Compounds containing 3'-OH and a 4'-OH are more potent. The addition of 5'-OH and the substitution of sugars abolishes the biological activity.

Gil *et al.* (1994) found that the anti-inflammatory activity of flavonoids is reduced if the free 6-OH or 7-OH groups are substituted with a methoxy (-OCH_3) group or a glucuronic acid moiety.

Chrysin, luteolin, apigenin, nepetin and hispidulin potentially have anti-inflammatory effect. The predicted potency for the inhibition of ICAM-1 expression is apigenin ≥ chrysin > luteolin (3'-OH) > hispidulin (6-OCH_3) > nepetin (3'-OH and 6-OCH_3). The prediction is different for the inhibition of LPS-stimulated TNF-α release: luteolin (3'- and 4'-OH) ≥ nepetin (3'- and 4'-OH) > apigenin ≥ chrysin ≥ hispidulin.

7.2.2.1.3 Antioxidant

To possess antioxidative activity, it is important to have hydroxyl groups in the A and B rings and the closed C ring (Shimoi *et al.*, 1996). The B ring with hydroxylation was reported to be the most important configuration for reactive oxygen species (ROS) (Burda and Oleszek, 2001; Sekher Pannala *et al.*, 2001) and reactive nitrogen species (RNS) (Haenen *et al.*, 1997; Kerry and Rice-Evans, 1999).

Flavonoids with a combination of the 2,3-double bond in the C ring and the 4-oxo function, and compounds with the central anthocyanidin C ring allowing stabilization of the aryloxyl radical, give high values of the Trolox equivalent antioxidant activity (TEAC) and show approximately the same potency (Rice-Evans *et al.*, 1996). A decrease of activity can occur via glycosylation or blockage/removal of 3-OH group. It is noted that the presence of 3-OH increases the antioxidant potential only when combined with the adjacent double bond in the C ring. The single 4'-OH does not contribute to bioactivity (e.g., TEACs of chrysin and apigenin are similar, 1.4 mM). Two hydroxyl groups in an *ortho*-position enhances the activity (e.g., TEAC of luteolin was higher than that of apigenin, 2.1 and 1.4 mM, respectively) but the addition of a third –OH group in the B ring is not

effective. On the other hand, the efficacy decreases with two –OH groups in a *meta*-arrangement. Pratt and Hudson (1990) suggested that the presence of 5'-OH and replacement of 4'-OH by a methoxyl group in the B ring suppress antioxidant activity. Interestingly, Mathiesen *et al.* (1997) reported that a molecule loses its scavenging ability when its configuration is changed from 6'-OH/4'-OCH_3 to 6'-OCH_3/4'-OH.

All seven flavonoids in *C. petasites* are therefore potentially antioxidant and the predicted activity is nepetin ≥ luteolin > hispidulin > apigenin = chrysin > naringenin > hesperetin.

7.2.2.2 Phenolic acids

Three phenolic acids, one hydroxybenzoic acid (i.e., vanillic acid) and two hydroxycinnamic acids (i.e., 4-coumaric and ferulic acids), were found in *C. petasites* and their structures are shown in Fig. 7.3.

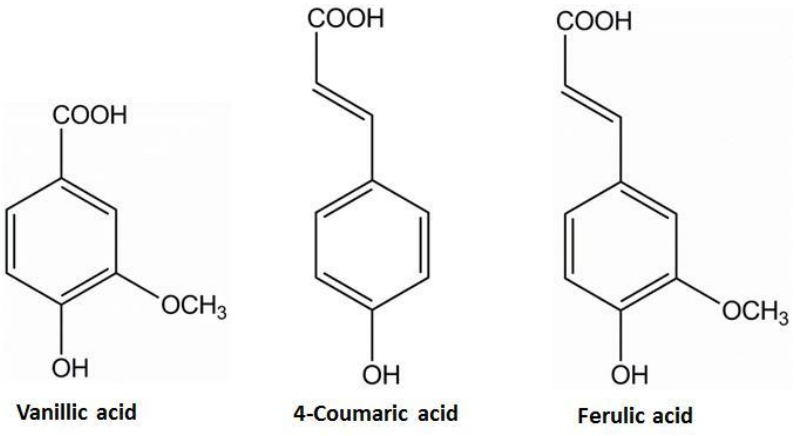


Fig. 7.3 Chemical structures of naturally-occurring phenolic acids in *C. petasites*.

Phenolic acids are well-known for their antioxidant activity which depends on the number of hydroxyl groups in the molecule (Rice-Evans *et al.*, 1996). For hydroxybenzoic acids, a single –OH group in the *ortho*- and *para*-positions confers no antioxidant activity, whereas an -OH at the *meta*-position does. The addition of a methylene group between the phenolic ring and the carboxylate group, or

dihydroxylation at *ortho*- and *meta*-positions to the carboxylate group, can result in an increase of antioxidant activity.

Hydroxycinnamic acids with an ethylene group between the phenolic ring and the carboxylate group and a *p*-OH (i.e., 4-coumaric acid with TEAC of 2.2 mM) possess stronger efficacy than those with *o*- or *m*-OH. Glycosylation of the –COOH group does not affect activity, whereas 3-OCH_3 substitution enhances it (i.e., ferulic acid with TEAC of 1.9 mM). It is noted that 3-OCH_3 has little influence on the activity of vanillic acid (TEAC of 1.43 mM). Although Rice-Evans *et al.* (1996) showed that 4-coumaric acid, which has a relatively high TEAC, shows stronger antioxidative effect than ferulic acid, Sekher Pannala *et al.* (2001) reported that the TEAC of 4-coumaric acid was lower (0.53 and 1.18 mM for 4-coumaric and ferulic acids, respectively). As mentioned above, 3-OCH_3 can increase the antioxidant ability meaning that ferulic acid is likely to be more active than 4-coumaric acid.

An investigation on $ONOO^-$ scavenging activity concluded that the number of –OCH_3 or –OH groups present are important (Kang *et al.*, 2009). If these functional groups are present in the same position between hydroxybenzoic acid and hydroxycinnamic acid, the latter shows higher efficacy resulting from stabilization of aryloxyl radicals after H-donation.

The order of effectiveness in antioxidant activities is deduced to be: ferulic acid > 4-coumaric acid > vanillic acid.

7.3 Conclusions

Based on the published effective concentrations, the measured skin uptakes, and on reported chemical structure-activity relationships, the four lead active compounds in *C. petasites*, vanillic acid, verbascoside, nepetin and hispidulin, are identified as plausible candidates responsible for the efficacy of this plant used in Thai traditional medicine. Antimicrobial, anti-inflammatory and antioxidant activities are well-supported by the data obtained and the information in the literature, and justify the further development of better and more efficient topical vehicles for these actives.

7.4 Thesis Conclusions

In this thesis, *Acanthus ebracteatus* Vahl and *Clerodendrum petasites* S. Moore were investigated. The goals were to identify and characterise their topically active substances, to optimise suitable topical formulations, and to determine the topical bioavailability of the active compounds. Overall, the results support the uses of these plants in Thai traditional medicine and contribute towards the establishment of guidelines for topical delivery systems for natural products.

Dried samples of each plant were treated with ethanolic extraction and the compounds obtained were subsequently separated by liquid-liquid partitioning and column chromatography. These ethanolic extracts and fractionations yielded two compounds (i.e., vanillic acid and verbascoside) from *A. ebracteatus* and eleven from *C. petasites*: vanillic acid, 4-coumaric acid, ferulic acid, verbascoside, nepetin, luteolin, apigenin, hispidulin, chrysin, naringenin, and hesperetin. Vanillic acid in *A. ebracteatus* and all compounds except apigenin and hispidulin in *C. petasites* are reported for the first time.

Because *A. ebracteatus* mainly contains glycosides and has fewer phenolic compounds, *C. petasites* was chosen as the lead plant to further optimise topical formulations and for the skin permeation studies. Of the eleven naturally-occurring compounds detected in *C. petasites*, vanillic acid, verbascoside, nepetin, apigenin and hispidulin were quantified. A predominant component was hispidulin, present at 39 µmol/g (1.2% w/w) in the dried ethanolic extract.

In vitro percutaneous penetration studies using Franz diffusion cells and abdominal pig skin were performed to determine the topical absorption of the principal actives in *C. petasites* (CP) and its formulations. The preliminary results following a 24-hour application of either a 50% w/w CP paste or a 50 $mg \cdot mL^{-1}$ CP solution (ethanol/water; 50:50 v/v) showed that delivery of most of the components was greater from the latter formulation. Hispidulin was measurably absorbed within 3 hours; vanillic acid and nepetin were detectable after 6 hours. Verbascoside was detected only in the outer SC, its large molecular weight (624 Da) and relatively high polarity (octanol-water partition coefficient, log P = -0.03), making this compound a poor skin penetrant.

An oil-in-water (o/w) cream and an o/w lotion each containing 10% w/w *C. petasites* extract were formulated with a view of improving skin permeability of vanillic acid, verbascoside, nepetin and hispidulin. These formulations contained propylene glycol and

glycerol, the surfactants Tween 60 and Span 60, and two emollients, glycerol monostearate-self emulsifier and mineral oil. A secondary objective was to avoid the use of ethanol as this co-solvent may alter the barrier function and irritate the skin. The application period was 6 hours to allow for comparison with subsequent *in vivo* experiments in human volunteers. Hispidulin penetrated in the greatest amount followed by vanillic acid and nepetin. Verbascoside only distributed into the stratum corneum (SC) and was not percutaneously absorbed. The *in vitro* permeation data for the four compounds showed no difference between the cream and lotion.

The relatively non-invasive method of tape stripping was employed to assess the skin uptake of the four compounds *in vivo* in humans. The methodology was robust and effective, resulting in low inter-subject variability. The SC concentration versus depth profiles of hispidulin and nepetin were similar in form to many other topical drugs that have been studied in a similar way. It could be seen that the partitioning of these compounds into the SC from the cream and lotion was comparable. The uptake of vanillic acid was much lower, while that of verbascoside was below the limit of its quantification (LOQ).

While extrapolation from the *in vitro* experiments using pig skin to the *in vivo* human measurements is difficult, the comparable SC levels observed in the two studies suggest that the former are useful, at least for preliminary formulation development work. It is worth noting that the cream and lotion vehicle elicited no adverse skin effects themselves.

In vitro release tests with a polysulfone membrane pre-soaked with the receptor solution buffer proved an adequate approach to discriminate the liberation of vanillic acid and verbascoside from the cream and lotion formulations containing 10% w/w CP extracts. Despite its poor ability to penetrate to the skin, verbascoside was efficiently released from both vehicles suggesting its potential usefulness as a skin surface-active drug (e.g., as an antimicrobial). This same approach, however, was not useful for hispidulin and nepetin, both of which were not released efficiently from the formulations, a reflection of a low leaving tendency into an essentially aqueous medium. An alternative membrane and/or receptor solution would need to be considered, therefore, for these more lipophilic compounds.

The potential biological activities of the four selected compounds in *C. petasites* were assessed based upon (i) the quantities taken up into the skin in the *in vitro* and *in vivo*

experiments, and (ii) the established chemical structure-bioactivity relationships reported in the literature. Comparison between these two sets of information suggests that formulations containing *C. petasites* extracts may possess antimicrobial, anti-inflammatory and antioxidant activities, that underpin the use of such a traditional Thai medicine to treat diseases such as rash, abscess and urticaria. The possible use of the extract as a topical antimicrobial would be novel.

With respect to physical stability of the formulations, the cream and lotion bases without plant extracts were stable for at least 1 month under accelerated conditions at 30°C and 75%RH (as recommended for Thailand by the World Health Organization (WHO)). These stability tests were conducted at the Center of Applied Thai Traditional Medicine, Faculty of Medicine Siriraj Hospital in Thailand. However, the formulations containing the plant extracts were unstable under the same stress condition. The plant extracts in the lotion separated more easily from the base than in the cream. Nevertheless, the separation was reversible by shaking. Formulation reproducibility was shown by consistent viscosity profiles between different batches of the two formulations (see Appendix 14).

Finally, in terms of next steps, it is clear that both lotion and cream formulations have positive attributes in terms of the delivery of the actives, but that the two vehicles have stability problems, which will limit their practical utility. This is clearly an area in which further work is required. With respect to verbascoside, it has been suggested that a liposomal formulation might enhance the accumulation of this compound in the SC (Sinico *et al.*, 2008), and provide a more stable product. There are no doubt other formulation approaches, perhaps involving less complex and less expensive excipients than those needed for a liposomal vehicle, which might also be examined.

References

Acanthus: Ngueak-Plaa-Mo [encyclopedia online]. In The encyclopedia of plants in Thailand. 2009. Forest Herbarium (BKF), Department of National Parks, Wildlife and Plant Conservation, Thailand. [accessed October 2012]. Available from: http://web3.dnp.go.th/botany/detail.aspx?words=%E0%CB%A7%D7%CD%A1%BB%C5%D2%CB%C1%CD&typeword=group.

Alberti, I., Kalia, Y.N., Naik, A., Bonny, J.D., Guy, R.H., 2001. *In vivo* assessment of enhanced topical delivery of terbinafine to human stratum corneum. J Control Release. 71(3), 319-327.

Albery, W.J., Hadgraft, J., 1979. Percutaneous absorption: *in vivo* experiments. J Pharm Pharmacol. 31(3), 140-147.

Alcaraz, L.E., Blanco, S.E., Puig, O.N., Tomas, F., Ferretti, F.H., 2000. Antibacterial activity of flavonoids against methicillin-resistant *Staphylococcus aureus* strains. J Theor Biol. 205(2), 231-240.

ALOGPS 2.1 algorithm [database on the internet]. Virtual Computational Chemistry Laboratory; 2001. Available from: http://146.107.217.178/lab/alogps/.

Anderson, R.L., Cassidy, J.M., 1973. Variation in physical dimensions and chemical composition of human stratum corneum. J Invest Dermatol. 61(1), 30-32.

Arct, J., Oborska, A., Mojski, M., Binkowska, A., Swidzikowska, B., 2002. Common cosmetic hydrophilic ingredients as penetration modifiers of flavonoids. Int J Cosmet Sci. 24(6), 357-366.

Arct, J., Pytkowska, K., 2008. Flavonoids as components of biologically active cosmeceuticals. Clin Dermatol. 26(4), 347-357.

Barry, B.W., 1988. Topical preparation, In: Aulton, M.E., editor. *Pharmaceutics: The science of dosage form design*. Churchill Livingstone, UK.

Boddé, H.E., Kruithof, M.A.M., Brussee, J., Koerten, H.K., 1989. Visualisation of normal and enhanced $HgCl_2$ transport through human skin *in vitro*. Int J Pharm. 53(1), 13-24.

Bonina, F., Lanza, M., Montenegro, L., Puglisi, C., Tomaino, A., Trombetta, D., Castelli, F., Saija, A., 1996. Flavonoids as potential protective agents against photo-oxidative skin damage. Int J Pharm. 145(1–2), 87-94.

Brian, K.R., Walters, K.A., 1993. Molecular modelling of skin permeation enhancement by chemical agents, In: Walters, K.A., Hadgraft, J.H., (Eds.). *Pharmaceutical skin penetration enhancement*. Marcel Dekker, New York. pp. 389-427.

Bunyapraphatsara, N., Jutiviboonsuk, A., Sornlek, P., Therathanathorn, W., Aksornkaew, S., Fong, H., Pezzuto, J., Kosmeder, J., 2003. Pharmacological studies of plants in the mangrove forest. Thai Journal of Phytopharmacy. 10(2), 1-12.

Burda, S., Oleszek, W., 2001. Antioxidant and antiradical activities of flavonoids. J Agric Food Chem. 49(6), 2774-2779.

Celiz, G., Daz, M., Audisio, M.C., 2011. Antibacterial activity of naringin derivatives against pathogenic strains. J Appl Microbiol. 111(3), 731-738.

Chemspider [database on the internet]. Royal Society of Chemistry (RSC). Available from: http://www.chemspider.com/.

Cimino, F., Saija, A., 2005. Flavonoids in skin cancer prevention. Curr Top Nutraceut Res. 3(4), 243-258.

Clavin, M., Gorzalczany, S., Macho, A., Munoz, E., Ferraro, G., Acevedo, C., Martino, V., 2007. Anti-inflammatory activity of flavonoids from *Eupatorium arnottianum*. J Ethnopharmacol. 112(3), 585-589.

Cleek, R.L., Bunge, A.L., 1993. A new method for estimating dermal absorption from chemical exposure. 1. General approach. Pharm Res. 10(4), 497-506.

Clerodendrum petasites S. Moore [database on the internet]. The International Plant Names Index (IPNI); 2005 [accessed October 2012]. Available from: http://www.ipni.org/ipni/idPlantNameSearch.do;jsessionid=CE5E067923B8D8124E22786A8BA23391?id=862308-1&back_page=%2Fipni%2FeditAdvPlantNameSearch.do%3Bjsessionid%3DCE5E067923B8D8124E22786A8BA23391%3Ffind_infragenus%3D%26find_isAPNIRecord%3Dtrue%26find_geoUnit%3D%26find_includePublicationAuthors%3Dtrue%26find_addedSince%3D%26find_family%3D%26find_genus%3Dclerodendrum%26find_sortByFamily%3Dtrue%26find_isGCIRecord%3Dtrue%26find_infrafamily%3D%26find_rankToReturn%3Dall%26find_publicationTitle%3D%26find_authorAbb.

Crank, J., 1975. The mathematics of diffusion. Clarendon Press, Oxford.

Cross, S.E., Roberts, M.S., 1993. Subcutaneous absorption kinetics of interferon and other solutes. J Pharm Pharmacol. 45, 606-609.

Delaquis, P., Stanich, K., Toivonen, P., 2005. Effect of pH on the inhibition of *Listeria* spp. by vanillin and vanillic acid. J Food Prot. 68(7), 1472-1476.

Diwan, P.V.R., Sitaramam, B.S., Ramakrishna, S., Ranghavan, K.V., inventors, 2001. Counsel of Scientific and Industrial Research, assignee. Herbal formulation useful as a therapeutic and cosmetic applications for the treatment of general skin disorders. USA patent US6200570.

Effendy, I., Maibach, H.I., 1996. Detergent and skin irritation. Clin Dermatol. 14(1), 15-21.

Elias, P.M., 1981. Epidermal lipids, membranes, and keratinization. Int J Dermatol. 20(1), 1-19.

Elias, P.M., 1983. Epidermal lipids, barrier function, and desquamation. J Invest Dermatol. 80 Suppl, 44s-49s.

Esposito, E., Dal Toso, R., Pressi, G., Bramanti, P., Meli, R., Cuzzocrea, S., 2010. Protective effect of verbascoside in activated C6 glioma cells: possible molecular mechanisms. Naunyn Schmiedebergs Arch Pharmacol. 381(1), 93-105.

Fluhr, J.W., Kao, J., Jain, M., Ahn, S.K., Feingold, K.R., Elias, P.M., 2001. Generation of free fatty acids from phospholipids regulates stratum corneum acidification and integrity. J Invest Dermatol. 117(1), 44-51.

Gerritsen, M.E., Carley, W.W., Ranges, G.E., Shen, C.P., Phan, S.A., Ligon, G.F., Perry, C.A., 1995. Flavonoids inhibit cytokine-induced endothelial cell adhesion protein gene expression. Am J Pathol. 147(2), 278-292.

Ghafourian, T., Samaras, E.G., Brooks, J.D., Riviere, J.E., 2010. Validated models for predicting skin penetration from different vehicles. Eur J Pharm Sci. 41(5), 612-616.

Gil, B., Sanz, M.J., Terencio, M.C., Ferrandiz, M.L., Bustos, G., Paya, M., Gunasegaran, R., Alcaraz, M.J., 1994. Effects of flavonoids on *Naja naja* and human recombinant synovial phospholipases A_2 and inflammatory responses in mice. Life Sci. 54(20), PL333-338.

Guidance for industry: Nonsterile semisolid dosage forms [database on the internet]. U.S. Department of Health and Human Services, Food and Drug Administration, Center for Drug Evaluation and Research (CDER); 1997 [accessed March 2013]. Available from: http://www.fda.gov/downloads/Drugs/Guidances/UCM070930.pdf.

Guide to the mangroves of Singapore [database on the internet]. Raffles Museum of Biodiversity Research, The National University of Singapore & The Singapore Science Centre; 2001 [accessed October 2012]. Available from: http://mangrove.nus.edu.sg/guidebooks/text/1045.htm.

Guy, R.H., 2010. Predicting the rate and extent of fragrance chemical absorption into and through the skin. Chem Res Toxicol. 23(5), 864-870.

Hachem, J.P., Crumrine, D., Fluhr, J., Brown, B.E., Feingold, K.R., Elias, P.M., 2003. pH directly regulates epidermal permeability barrier homeostasis, and stratum corneum integrity/cohesion. J Invest Dermatol. 121(2), 345-353.

Hadgraft, J.W., Somers, G.F., 1956. Percutaneous absorption. J Pharm Pharmacol. 8(1), 625-634.

Haenen, G.R.M.M., Paquay, J.B.G., Korthouwer, R.E.M., Bast, A., 1997. Peroxynitrite scavenging by flavonoids. Biochem Biophys Res Commun. 236(3), 591-593.

Hall-Manning, T.J., Holland, G.H., Rennie, G., Revell, P., Hines, J., Barratt, M.D., Basketter, D.A., 1998. Skin irritation potential of mixed surfactant systems. Food Chem Toxicol. 36(3), 233-238.

Harvell, J.D., Tsai, Y.-C., Maibach, H.I., Gay, R., Gordon, V.C., Miller, K., Mun, G.C., 1994. An *in vivo* correlation with three *in vitro* assays to assess skin irritation potential. Cutan Ocul Toxicol. 13(2), 171-183.

Hawkins, G.S., Reifenrath, W.G., 1986. Influence of skin source, penetration cell fluid, and partition coefficient on *in vitro* skin penetration. J Pharm Sci. 75(4), 378-381.

Hazekamp, A., Verpoorte, R., Panthong, A., Hazekamp, A., Verpoorte, R., Panthong, A., 2001. Isolation of a bronchodilator flavonoid from the Thai medicinal plant *Clerodendrum petasites*. J Ethnopharmacol. 78(1), 45-49.

He, J., Hu, X.P., Zeng, Y., Li, Y., Wu, H.Q., Qiu, R.Z., Ma, W.J., Li, T., Li, C.Y., He, Z.D., 2011. Advanced research on acteoside for chemistry and bioactivities. J Asian Nat Prod Res. 13(5), 449-464.

Heisig, M., Lieckfeldt, R., Wittum, G., Mazurkevich, G., Lee, G., 1996. Non steady-state descriptions of drug permeation through stratum corneum. I. The biphasic brick-and-mortar model. Pharm Res. 13(3), 421-426.

Herkenne, C., Naik, A., Kalia, Y.N., Hadgraft, J., Guy, R.H., 2007. Ibuprofen transport into and through skin from topical formulations: *in vitro-in vivo* comparison. J Invest Dermatol. 127(1), 135-142.

Higuchi, T., 1961. Rate of release of medicaments from ointment bases containing drugs in suspension. J Pharm Sci. 50, 874-875.

Hokputsa, S., Harding, S., Inngjerdingen, K., Jumel, K., Michaelsen, T., Heinze, T., Koschella, A., Paulsen, B., 2004. Bioactive polysaccharides from the stems of the Thai medicinal plant *Acanthus ebracteatus*: their chemical and physical features. Carbohyd Res. 339(4), 753-762.

Holleran, W.M., Takagi, Y., Imokawa, G., Jackson, S., Lee, J.M., Elias, P.M., 1992. beta-Glucocerebrosidase activity in murine epidermis: characterization and localization in relation to differentiation. J Lipid Res. 33(8), 1201-1209.

Hotchkiss, S.A.M., 1998. Dermal metabolism, In: Roberts, M.S., Walters, K.A., (Eds.). *Dermal absorption and toxicity assessment*. Marcel Dekker, New York.

Hsiao, J., Lin, M., 1995. A chemotaxonomic study of essential oils from the leaves of genus *Clerodendrum* (Verbenaceae) native in Taiwan. Bot Bull Acad Sinica. 36, 247-251.

Ishikawa, S., Matsumura, Y., Katoh-Kubo, K., Tsuchido, T., 2002. Antibacterial activity of surfactants against *Escherichia coli* cells is influenced by carbon source and anaerobiosis. J Appl Microbiol. 93(2), 302-309.

Kalia, Y.N., Alberti, I., Sekkat, N., Curdy, C., Naik, A., Guy, R.H., 2000. Normalization of stratum corneum barrier function and transepidermal water loss *in vivo*. Pharm Res. 17(9), 1148-1150.

Kanchanapoom, T., Kasai, R., Picheansoonthon, C., Yamasaki, K., 2001. Megastigmane, aliphatic alcohol and benzoxazinoid glycosides from *Acanthus ebracteatus*. Phytochemistry. 58(5), 811-817.

Kang, K.S., Tanaka, T., Cho, E.J., Yokozawa, T., 2009. Evaluation of the peroxynitrite scavenging activity of heat-processed ginseng. J Med Food. 12(1), 124-130.

Kerry, N., Rice-Evans, C., 1999. Inhibition of peroxynitrite-mediated oxidation of dopamine by flavonoid and phenolic antioxidants and their structural relationships. J Neurochem. 73(1), 247-253.

Ki, V., Rotstein, C., 2008. Bacterial skin and soft tissue infections in adults: A review of their epidemiology, pathogenesis, diagnosis, treatment and site of care. Can J Infect Dis Med Microbiol. 19(2), 173-184.

Kim, M.C., Kim, S.J., Kim, D.S., Jeon, Y.D., Park, S.J., Lee, H.S., Um, J.Y., Hong, S.H., 2011. Vanillic acid inhibits inflammatory mediators by suppressing NF-kappaB in lipopolysaccharide-stimulated mouse peritoneal macrophages. Immunopharmacol Immunotoxicol. 33(3), 525-532.

Klaiklay, S., 2009. Chemical constituents from the twigs of *Garcinia hombroniana*, the leaves of *Garcinia prainiana* and the roots of *Clerodendrum petasites* S. Moore. MSc. Thesis, Prince of Songkla University Thailand.

Korkina, L.G., Mikhal'chik, E., Suprun, M.V., Pastore, S., Dal Toso, R., 2007. Molecular mechanisms underlying wound healing and anti-inflammatory properties of naturally

occurring biotechnologically produced phenylpropanoid glycosides. Cell Mol Biol (Noisy-le-grand). 53(5), 84-91.

Koschella, A., Inngjerdingen, K., Paulsen, B.S., Morris, G.A., Harding, S.E., Heinze, T., 2008. Unconventional methyl galactan synthesized via the thexyldimethylsilyl intermediate: Preparation, characterization, and properties. Macromol Biosci. 8(1), 96-105.

Kushner, J.t., Blankschtein, D., Langer, R., 2007. Evaluation of the porosity, the tortuosity, and the hindrance factor for the transdermal delivery of hydrophilic permeants in the context of the aqueous pore pathway hypothesis using dual-radiolabeled permeability experiments. J Pharm Sci. 96(12), 3263-3282.

Lambers, H., Piessens, S., Bloem, A., Pronk, H., Finkel, P., 2006. Natural skin surface pH is on average below 5, which is beneficial for its resident flora. Int J Cosmet Sci. 28(5), 359-370.

Lansdown, A.B., Grasso, P., 1972. Physico-chemical factors influencing epidermal damage by surface active agents. Br J Dermatol. 86(4), 361-373.

Laupattarakasem, P., Houghton, P., Hoult, J., Itharat, A., 2003. An evaluation of the activity related to inflammation of four plants used in Thailand to treat arthritis. J Ethnopharmacol. 85(2-3), 207-215.

Lee, J.S., Kim, J.W., Han, S.H., Chang, I.S., Kang, H.H., Lee, O.S., Oh, S.G., Suh, K.D., 2004. The stabilization of L-ascorbic acid in aqueous solution and water-in-oil-in-water double emulsion by controlling pH and electrolyte concentration. J Cosmet Sci. 55(1), 1-12.

Li, M.-Y., Xiao, Q., Pan, J.-Y., Wu, J., 2009. Natural products from semi-mangrove flora: source, chemistry and bioactivities. Nat. Prod. Rep. 26, 281-298.

Lin, J.W., Chiang, H.M., Lin, Y.C., Wen, K.C., 2008. Natural products with skin - whitening effects. J Food Drug Anal. 16(2), 1-10.

List of herbal medicinal products, 2006. National drug committee, Bangkok, Thailand.

Liu, H., Mou, Y., Zhao, J., Wang, J., Zhou, L., Wang, M., Wang, D., Han, J., Yu, Z., Yang, F., 2010. Flavonoids from *Halostachys caspica* and their antimicrobial and antioxidant activities. Molecules. 15(11), 7933-7945.

Lv, P.C., Li, H.Q., Xue, J.Y., Shi, L., Zhu, H.L., 2009. Synthesis and biological evaluation of novel luteolin derivatives as antibacterial agents. Eur J Med Chem. 44(2), 908-914.

Mahasiripanth, T., Hokputsa, S., Niruthisard, S., Bhattarakosol, P., Patumraj, S., 2012. Effects of *Acanthus ebracteatus* Vahl on tumor angiogenesis and on tumor growth in nude mice implanted with cervical cancer. Cancer Manag Res. 4, 269-279.

Marangon, A., Bock, U., Haltner, E. *In vitro* release testing for semisolid formulations [database on the internet]. Across Barriers GmbH; 2009 [accessed March 2013]. Available from: http://www.acrossbarriers.de/uploads/media/FCT08_SUPAC_SS.pdf.

Mathiesen, L., Malterud, K.E., Sund, R.B., 1997. Hydrogen bond formation as basis for radical scavenging activity: a structure-activity study of C-methylated dihydrochalcones from *Myrica gale* and structurally related acetophenones. Free Radic Biol Med. 22(1-2), 307-311.

McCarley, K.D., Bunge, A.L., 1998. Physiologically relevant one-compartment pharmacokinetic models for skin. 1. Development of models. J Pharm Sci. 87(4), 470-481.

McCarley, K.D., Bunge, A.L., 2000. Physiologically relevant two-compartment pharmacokinetic models for skin. J Pharm Sci. 89(9), 1212-1235.

Merianos, J.J., 1991. Quaternary ammonium antimicrobial compounds, *Disinfection, sterilization and preservation*. Lea & Febiger, Philadelphia, USA.

Michaels, A.S., Chandrasekaran, S.K., Shaw, J.E., 1975. Drug permeation through human skin: theory and *in vitro* experimental measurement. AIChE J. 21, 985-996.

Mitragotri, S., Anissimov, Y.G., Bunge, A.L., Frasch, H.F., Guy, R.H., Hadgraft, J., Kasting, G.B., Lane, M.E., Roberts, M.S., 2011. Mathematical models of skin permeability: an overview. Int J Pharm. 418(1), 115-129.

Murakami, A., Ohigashi, H., Koshimizu, K., 1994. Possible anti-tumour promoting properties of traditional Thai food items and some of their active constituents. Asia Pacific J Clin Nutr. 3, 185-191.

N'Dri-Stempfer, B., Navidi, W.C., Guy, R.H., Bunge, A.L., 2009. Improved bioequivalence assessment of topical dermatological drug products using dermatopharmacokinetics. Pharm Res. 26(2), 316-328.

National list of essential medicines: Ha-Rak [database on the internet]. Food and Drug Administration in Thailand (Thai FDA); [accessed October 2012]. Available from: http://www.nlem.in.th/medicine/herbal/list/637.

Okochi, H., Nakano, M., 2000. Preparation and evaluation of w/o/w type emulsions containing vancomycin. Adv Drug Deliv Rev. 45(1), 5-26.

Panthong, A., Kanjanapothi, D., Taesotikul, T., Wongcome, T., Reutrakul, V., 2003. Anti-inflammatory and antipyretic properties of *Clerodendrum petasites* S. Moore. J Ethnopharmacol. 85(1), 151-156.

Panthong, A., Kanjanapothi, D., Taylor, W., 1986. Ethnobotanical review of medicinal plants from Thai traditional books, Part I: Plants with anti-inflammatory, anti-asthmatic and antihypertensive properties. J Ethnopharmacol. 18(3), 213-228.

Pastore, S., Potapovich, A., Kostyuk, V., Mariani, V., Lulli, D., De Luca, C., Korkina, L., 2009. Plant polyphenols effectively protect HaCaT cells from ultraviolet C-triggered necrosis and suppress inflammatory chemokine expression. Ann N Y Acad Sci. 1171, 305-313.

Perron, N.R., Brumaghim, J.L., 2009. A review of the antioxidant mechanisms of polyphenol compounds related to iron binding. Cell Biochem Biophys. 53(2), 75-100.

Pichaensoonthon, C., Chaowalit, M., Jirawong, W., 2005. The explanation of traditional recipes: Osot-Phra-Na-Rai (โอสถพระนารายณ์). Amarin Printing and Publishing, Bangkok, Thailand.

Pongboonrot, S., 1965. Foreign-Thai medicine and materia medica. Kasem Bannakij, Bangkok, Thailand.

Potts, R.O., Guy, R.H., 1992. Predicting skin permeability. Pharm Res. 9(5), 663-669.

Poulsen, B.J., Young, E., Coquilla, V., Katz, M., 1968. Effect of topical vehicle composition on the *in vitro* release of fluocinolone acetonide and its acetate ester. J Pharm Sci. 57(6), 928-933.

Pratt, D., Hudson, B.J.F., 1990. Natural antioxidants not commercially exploited, In: Hudson, B.J.F., editor. *Food antioxidants*. Elsvier, Amsterdam.

Rajesh, K., Murugesan, S.K., Nanjaian, M., 2012. Multiple emulsions: A review. Int J Recent Adv Pharm Res. 2(1), 9-19.

Reddy, M.B., McCarley, K.D., Bunge, A.L., 1998. Physiologically relevant one-compartment pharmacokinetic models for skin. 2. Comparison of models when combined with a systemic pharmacokinetic model. J Pharm Sci. 87(4), 482-490.

Reddy, M.B., Stinchcomb, A.L., Guy, R.H., Bunge, A.L., 2002. Determining dermal absorption parameters *in vivo* from tape strip data. Pharm Res. 19(3), 292-298.

Reich, E., Schibli, A., 2007. High-performance thin-layer chromatography for the anlaysis of medicinal plants. Thieme Medical Publishers, New York.

Rice-Evans, C.A., Miller, N.J., Paganga, G., 1996. Structure-antioxidant activity relationships of flavonoids and phenolic acids. Free Radic Biol Med. 20(7), 933-956.

Robak, J., Gryglewski, R.J., 1996. Bioactivity of flavonoids. Pol J Pharmacol. 48(6), 555-564.

Rojanapo, W., Tepsuwan, A., Siripong, P., 1990. Mutagenicity and antimutagenicity of Thai medicinal plants. Basic Life Sci. 52, 447-452.

Rueda, R., 1993. The genus *Clerodendrum* (Verbenaceae) in Mesoamerica. Ann Missouri Bot Gard. 80, 870-890.

Russell, L.M., Wiedersberg, S., Delgado-Charro, M.B., 2008. The determination of stratum corneum thickness: an alternative approach. Eur J Pharm Biopharm. 69(3), 861-870.

Scheuplein, R.J., 1967. Mechanism of percutaneous absorption. II. Transient diffusion and the relative importance of various routes of skin penetration. J Invest Dermatol. 48(1), 79-88.

Schmid-Wendtner, M.H., Korting, H.C., 2006. The pH of the skin surface and its impact on the barrier function. Skin Pharmacol Physiol. 19(6), 296-302.

Scott, R.P.W. Thin layer chromatography (Chrom-Ed Series): The sulfuric acid spray [database on the internet]. Library4science.com, LLC.; 2000-2009 [accessed Jan 2013]. Available from: http://www.chromatography-online.org/TLC/Detection-and-Quantitatitation/Sulfuric-Acid-Spray.html.

Sea Holly [database on the internet]. 2001 [accessed October 2012]. Available from: http://www.naturia.per.sg/buloh/plants/sea_holly.htm.

Sekher Pannala, A., Chan, T.S., O'Brien, P.J., Rice-Evans, C.A., 2001. Flavonoid B-ring chemistry and antioxidant activity: fast reaction kinetics. Biochem Biophys Res Commun. 282(5), 1161-1168.

Shah, J.C., 1993. Analysis of permeation data: evaluation of the lag time method. Int J Pharm. 90(2), 161-169.

Shikanga, E.A., Combrinck, S., Regnier, T., 2010. South African Lippia herbal infusions: Total phenolic content, antioxidant and antibacterial activities. S Afr J Bot. 76(3), 567-571.

Shimoi, K., Masuda, S., Shen, B., Furugori, M., Kinae, N., 1996. Radioprotective effects of antioxidative plant flavonoids in mice. Mutat Res. 350(1), 153-161.

Shrivastava, N., Patel, T., 2007. *Clerodendrum* and Heathcare: An overview. Medicinal Aromatic Plant Sci Biotech. 1(1), 142-150.

Singharachai, C., Palanuvej, C., Kiyohara, H., Yamada, H., Ruangrungsi, N., 2011a. Pharmacognostic specification of five root species in Thai traditional medicine remedy: Ben-Cha-Lo-Ka-Wi-Chian. Phcog J. 3(21), 1-11.

Singharachai, C., Palanuvej, C., Kiyohara, H., Yamada, H., Ruangrungsi, N., 2011b. Safety evaluation of Thai traditional medicine remedy: Ben-Cha-Lo-Ka-Wi-Chian. J Health Res. 25(2), 83-90.

Sinico, C., Caddeo, C., Valenti, D., Fadda, A.M., Bilia, A.R., Vincieri, F.F., 2008. Liposomes as carriers for verbascoside: stability and skin permeation studies. J Liposome Res. 18(1), 83-90.

Sittiwet, C., Niamsa, N., Puangpronpitag, D., 2009. Antimicrobial activity of *Acanthus ebracteatus* Vahl. aqueous extract: The potential for skin infection treatment. Int J Biol Chem. 3, 95-98.

Somchaichana, J., Bunaprasert, T., Patumraj, S., 2012. *Acanthus ebracteatus* Vahl. ethanol extract enhancement of the efficacy of the collagen scaffold in wound closure: a study in a full-thickness-wound mouse model. J Biomed Biotechnol. 2012, 754527.

Sultana, N., Afolayan, A.J., 2007. A novel daucosterol derivative and antibacterial activity of compounds from *Arctotis arctotoides*. Nat Prod Res. 21(10), 889-896.

Thai traditional medical textbook: Paet-Ta-Ya-Saat-Song-Kror (แพทยศาสตร์สงเคราะห์), 2007. conservative (ฉบับอนุรักษ์) ed. The Rehabilitation Foundation for Thai Traditional Medicine and Ayurved Thamrong School, Bangkok, Thailand.

Thangthaisong, T., Caichompoo, W., Phadungkit, M., 2011. The ethnobotanical survey of plants used for treatment of bone disorders in Amphur Muang, Mahasarakram Province. Thai Pharm Health Sci J. 6(1), 7-11.

Thao-Yai-Mom [database on the internet]. Thailand Institute of Scientific and Technological Research (TISTR); [accessed October 2012]. Available from: http://www.tistr.or.th/sakaerat/Plant%20in%20Sakaerat/plant%20list/%E0%B9%80%E0%B8%97%E0%B9%89%E0%B8%B2%E0%B8%A2%E0%B8%B2%E0%B8%A2%E0%B8%A1%E0%B9%88%E0%B8%AD%E0%B8%A1.pdf.

Thongchai, W., Liawruangrath, B., Liawruangrath, S., 2007. High-performance liquid chromatographic determination of arbutin in skin-whitening creams and medicinal plant extracts. J Cosmet Sci. 58(1), 35-44.

Topical and transdermal drug products. 2009. *Pharmacopoeial Forum*: The United States Pharmacopeial Convention, Inc., pp. 750-764.

Tsuchiya, H., Sato, M., Miyazaki, T., Fujiwara, S., Tanigaki, S., Ohyama, M., Tanaka, T., Iinuma, M., 1996. Comparative study on the antibacterial activity of phytochemical flavanones against methicillin-resistant *Staphylococcus aureus*. J Ethnopharmacol. 50(1), 27-34.

Tungjitjaruen, S., 1977. Good drug recipes: Tam-ra-ya-wi-sade (ตำรายาวิเศษ). Five Pharmacists (ห้าเภสัชกร), Thailand.

Uchida, Y., Hara, M., Nishio, H., Sidransky, E., Inoue, S., Otsuka, F., Suzuki, A., Elias, P.M., Holleran, W.M., Hamanaka, S., 2000. Epidermal sphingomyelins are precursors for selected stratum corneum ceramides. J Lipid Res. 41(12), 2071-2082.

Uses of mangrove plant products: Traditional uses of mangrove plant products in India [database on the internet]. Bioinformatics Centre, National Institute of Oceanography, Dona Paula, Goa, India; 1998 [accessed October 2012]. Available from: http://www.niobioinformatics.in/mangroves/MANGCD/uses.htm#ebract.

Wagner, H., Kostka, K.H., Lehr, C.M., Schaefer, U.F., 2000. Drug distribution in human skin using two different *in vitro* test systems: comparison with *in vivo* data. Pharm Res. 17(12), 1475-1481.

Wertz, P.W., Miethke, M.C., Long, S.A., Strauss, J.S., Downing, D.T., 1985. The composition of the ceramides from human stratum corneum and from comedones. J Invest Dermatol. 84(5), 410-412.

Wilkes, G.L., Brown, I.A., Wildnauer, R.H., 1973. The biomechanical properties of skin. CRC Crit Rev Bioeng. 1(4), 453-495.

Williams, A.C., 2003. Transdermal and topical drug delivery. Pharmaceutical Press, London, UK.

Wuguo, D., Xingwang, F., Jilan, W., 1997. Flavonoids function as antioxidants: By scavenging reactive oxygen species or by chelating iron? Radiation Physics and Chemistry. 50(3), 271-276.

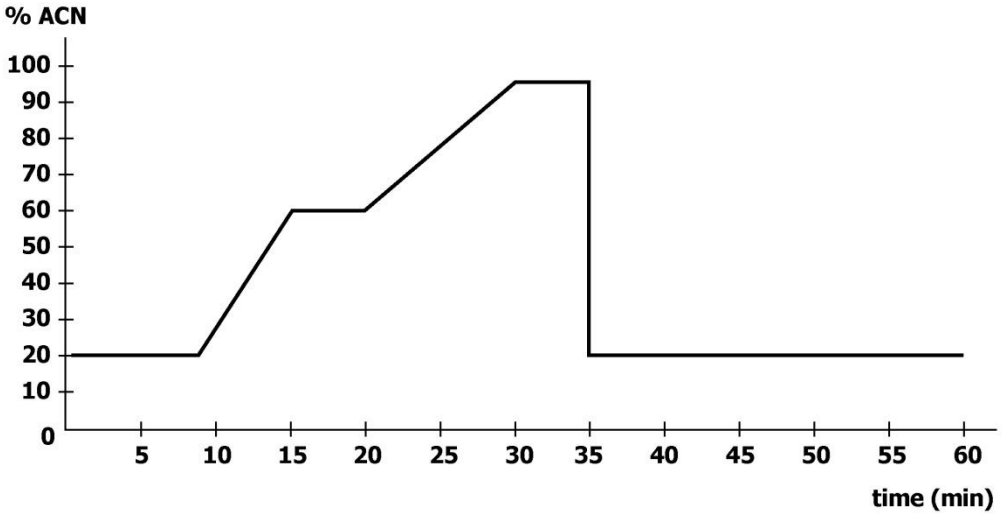
Xagorari, A., Papapetropoulos, A., Mauromatis, A., Economou, M., Fotsis, T., Roussos, C., 2001. Luteolin inhibits an endotoxin-stimulated phosphorylation cascade and proinflammatory cytokine production in macrophages. J Pharmacol Exp Ther. 296(1), 181-187.

Zatz, J.L., Segers, J.D., 1998. Techniques for measuring *in vitro* release from semisolids. Dissolution Technol. 5(2), 3-13.

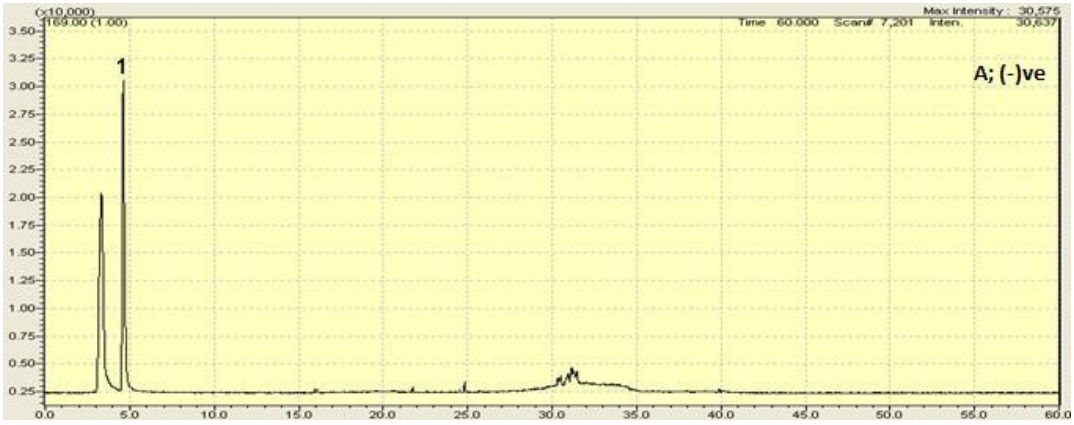
Appendices

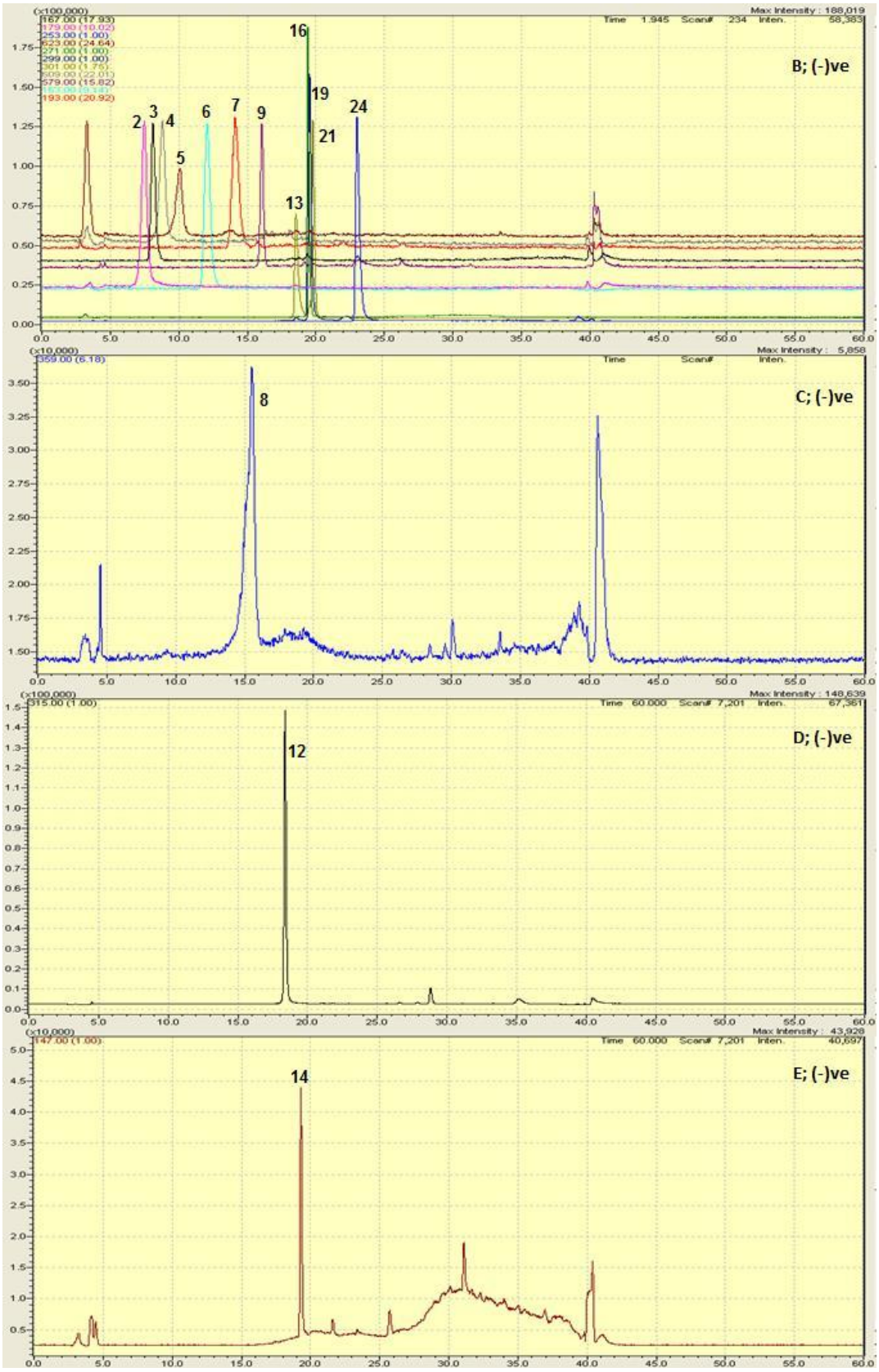
Appendix 1: MS chromatograms of phenolic standards

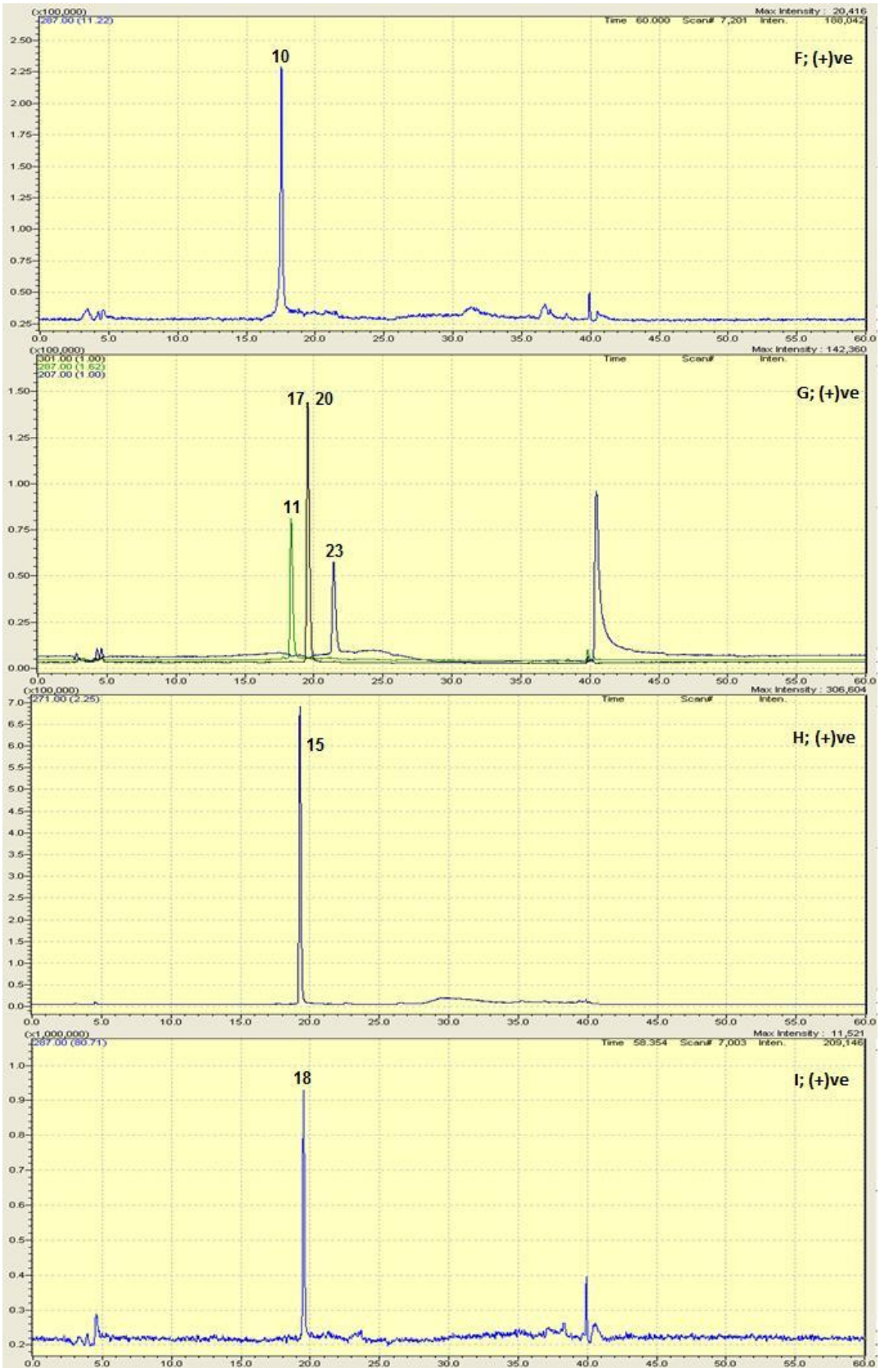
Appendix 1.1 Optimised method for HPLC-MS using acetonitrile and 0.1% aqueous acetetic acid.



Appendix 1.2 MS chromatograms of twenty four phenolic standard markers at concentrations of 1 µg·mL^{-1} dissolved in methanol (20 µL) in selected negative ion mode (A-E) and positive ion mode (F-J). Peak identification, mass to charge ratios (m/z) and retention times (t_R) are given in Table 3.4, Chapter 3.




B; (-)ve
C; (-)ve
D; (-)ve
E; (-)ve
2
3
4
5
6
7
9
13
16
19
21
24
8
12
14

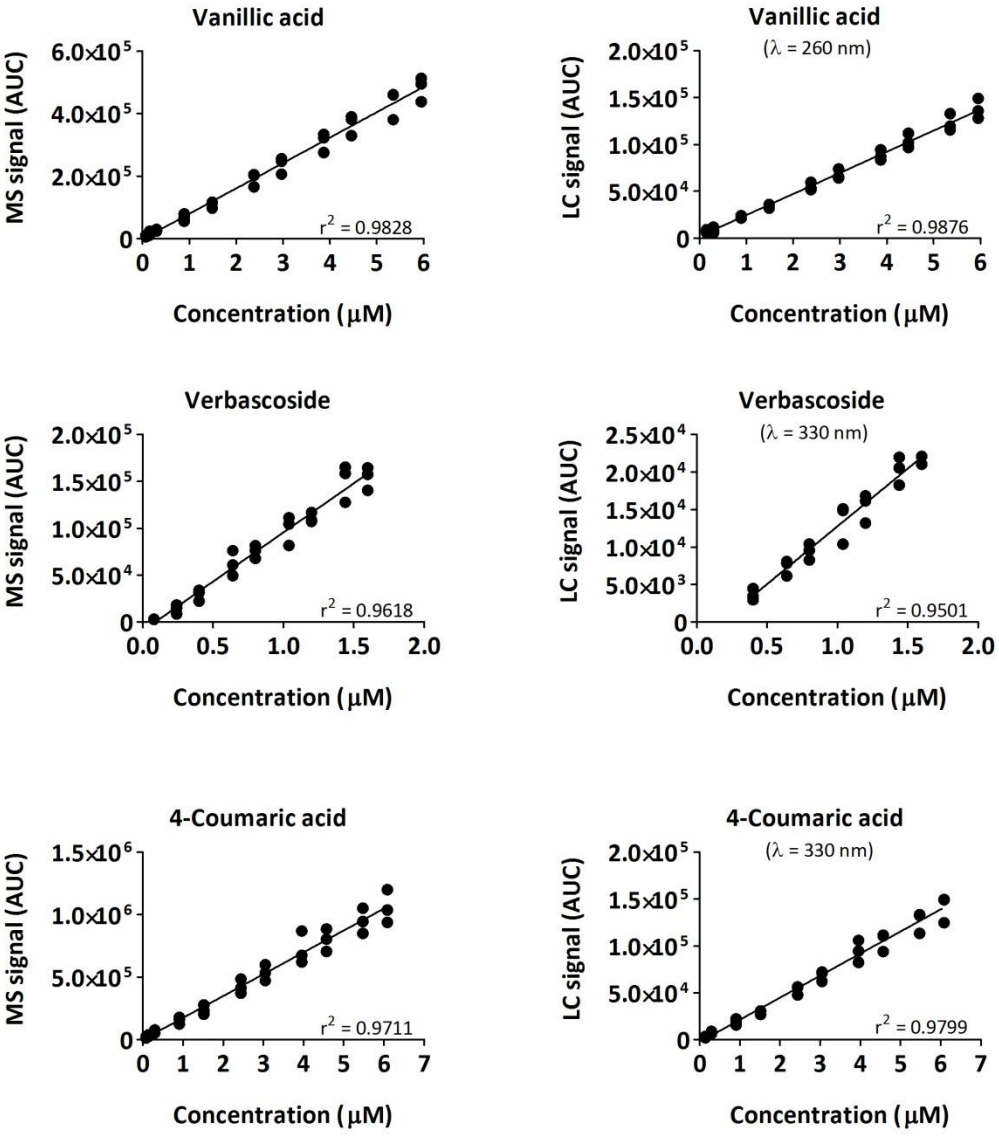

F; (+)ve
10
G; (+)ve
17, 20
11
23
H; (+)ve
15
I; (+)ve
18


(x100,000)
Max Intensity : 90,857
Time 2.020 Scan# 243 Inten. 423,983
J; (+)ve
22
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
0.0
5.0
10.0
15.0
20.0
25.0
30.0
35.0
40.0
45.0
50.0
55.0
60.0

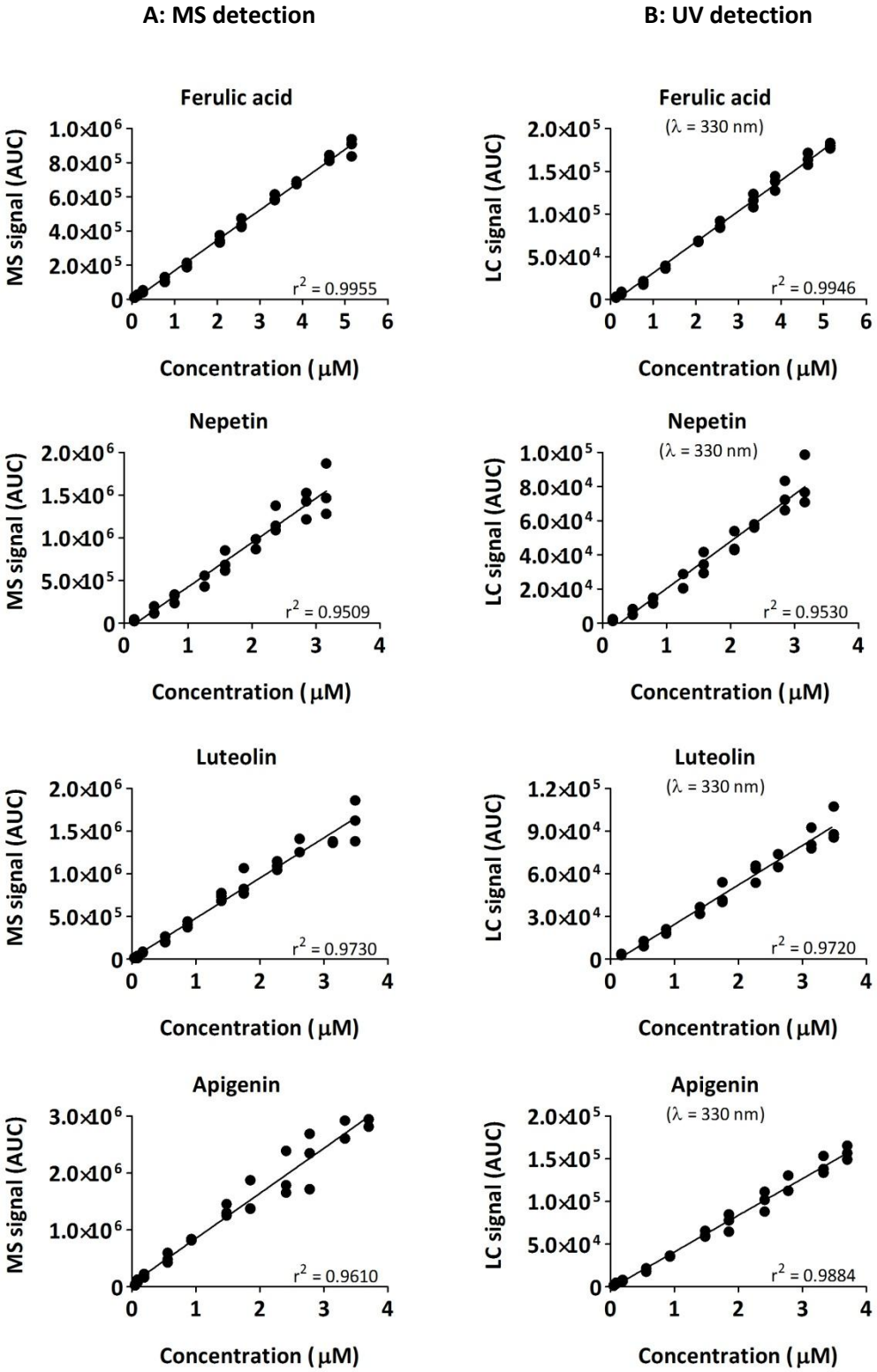
Appendix 2: Calibration curves of phenolic standards

Appendix 2.1 Calibration curves of vanillic acid, verbascoside and 4-coumaric acid by MS (Panel A) and UV (Panel B) detections; a concentration range of 0.01-6 µM in methanol, n = 3.

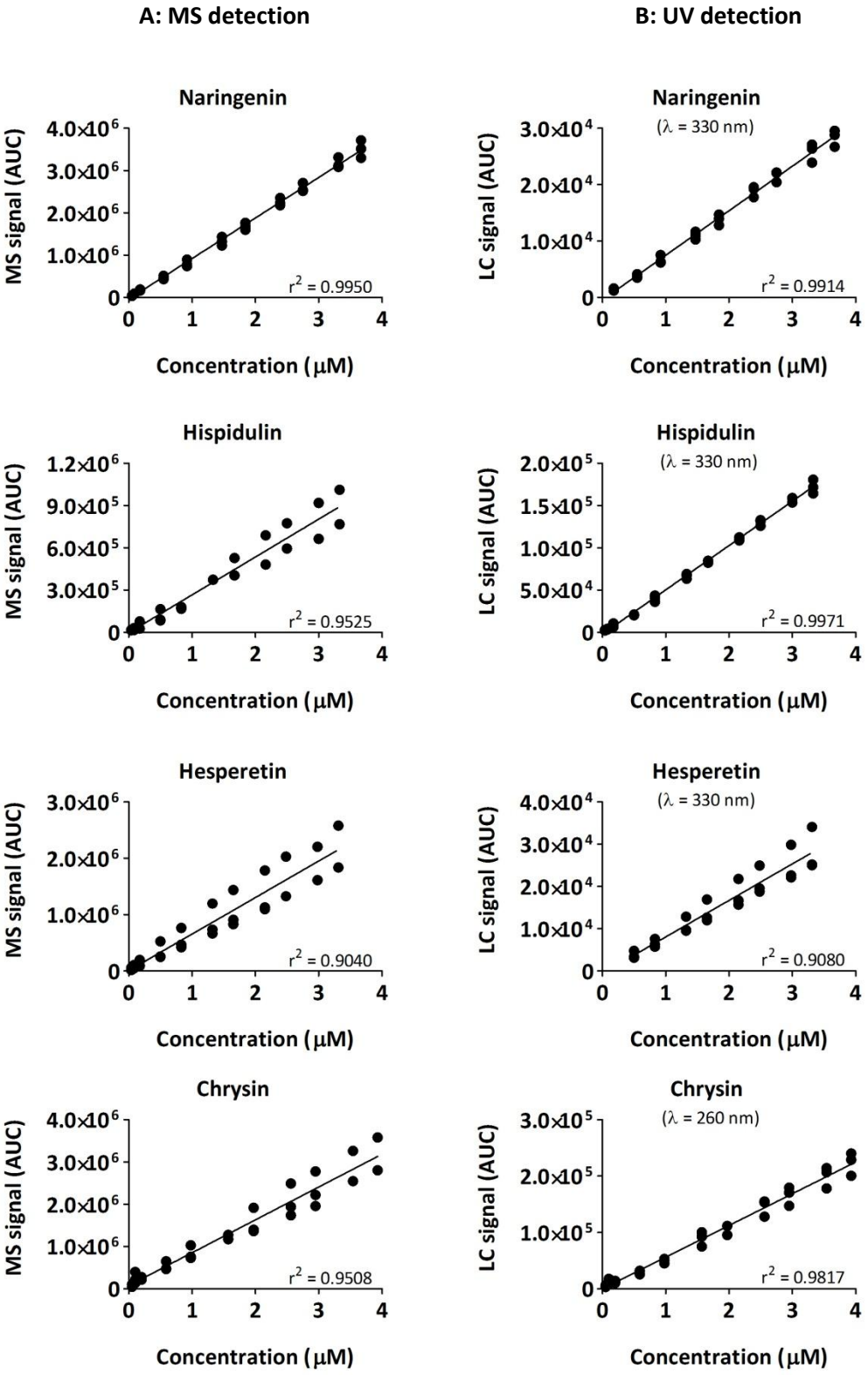
A: MS detection **B: UV detection**



Appendix 2.2 Calibration curves of ferulic acid, nepetin, luteolin and apigenin by MS (Panel A) and UV (Panel B) detections; a concentration range of 0.01-6 µM in methanol, n = 3.

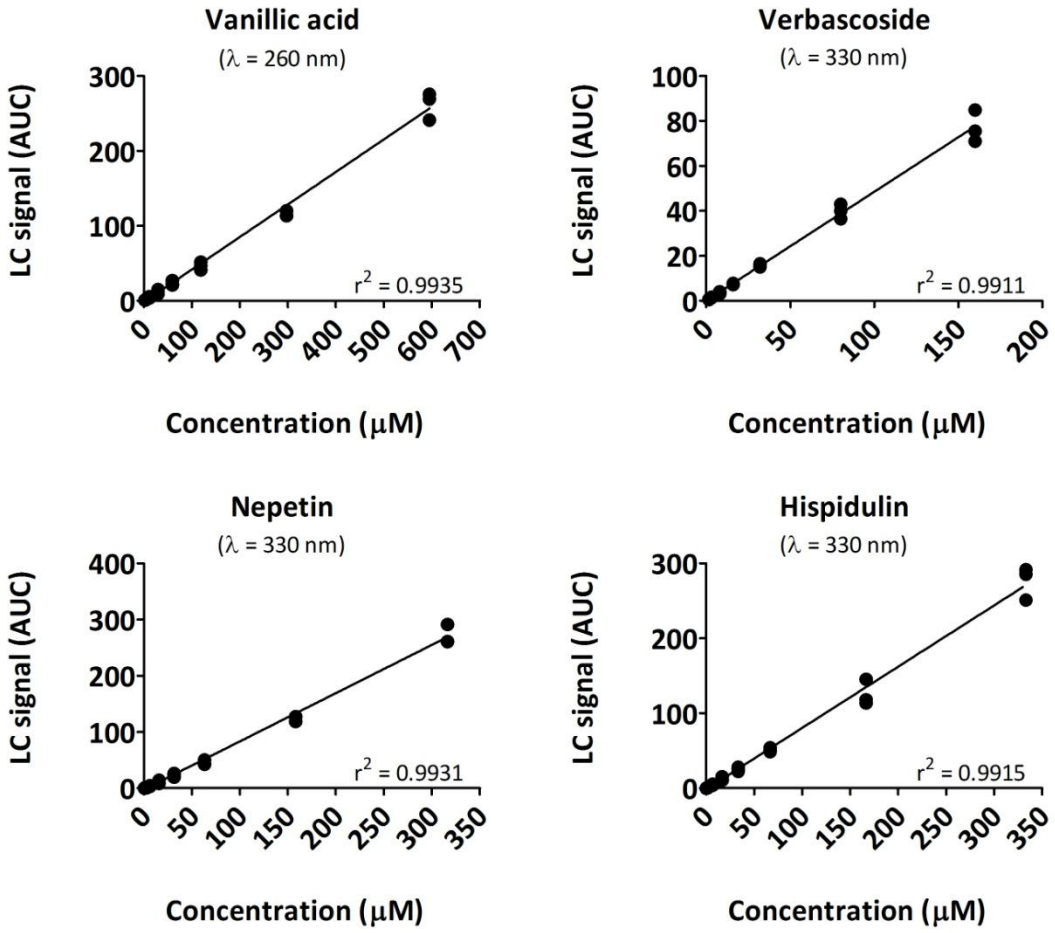


Appendix 2.3 Calibration curves of naringenin, hispidulin, hesperetin and chrysin by MS (Panel A) and UV (Panel B) detections; a concentration range of 0.01-6 µM in methanol, n=3.



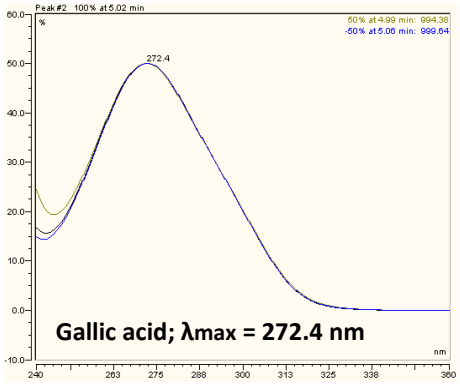
Appendix 3: Calibration curves of phenolic standards by PDA detection

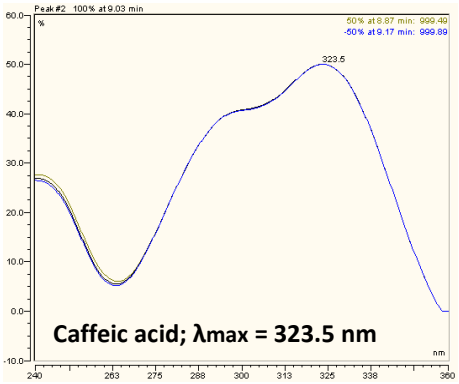
Appendix 3.1 Calibration curves of vanillic acid, verbascoside, nepetin, and hispidulin by PDA detection; a concentration range of 0.08-600 µM in methanol, n = 3.

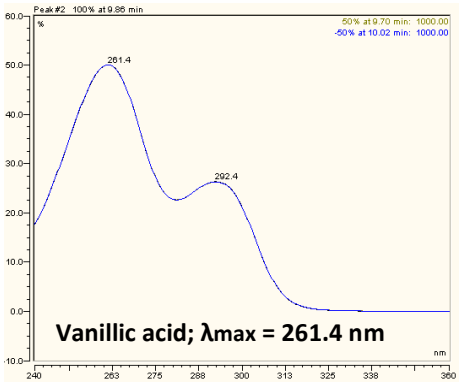


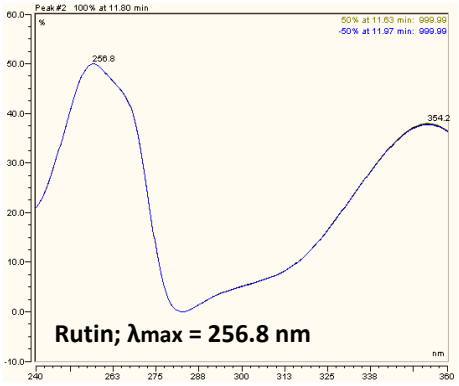
Appendix 4: PDA spectra of phenolic standards

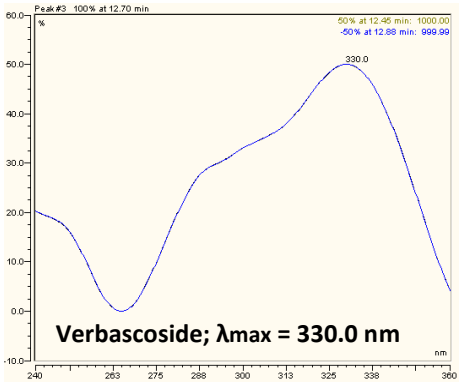
Appendix 4.1 PDA spectra of gallic acid, caffeic acid, vanillic acid, rutin, verbascoside and 4-coumaric acid; a wavelength scanning range of 246-360 nm, 0.1 mg·mL^{-1} in methanol.

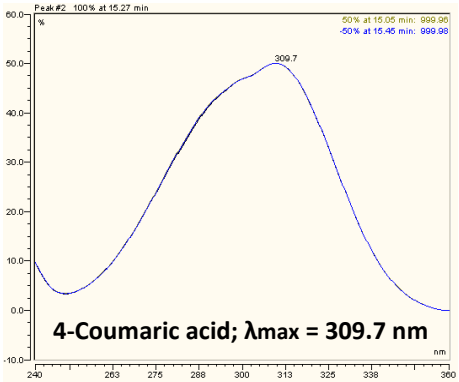




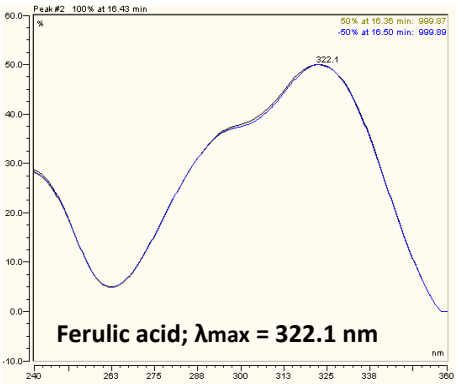


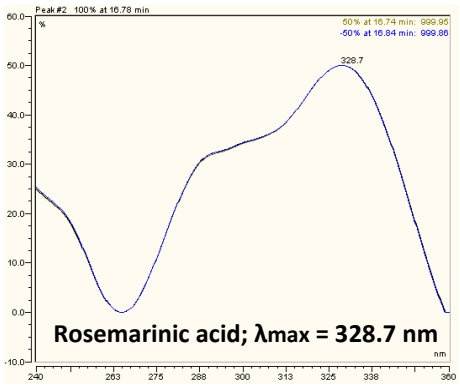


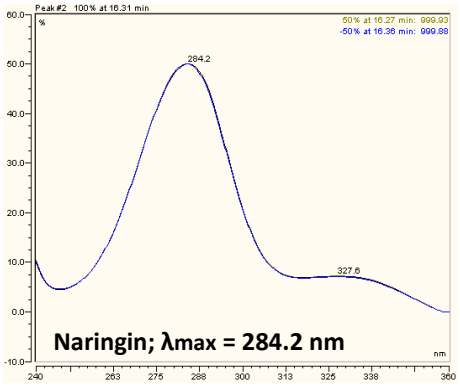


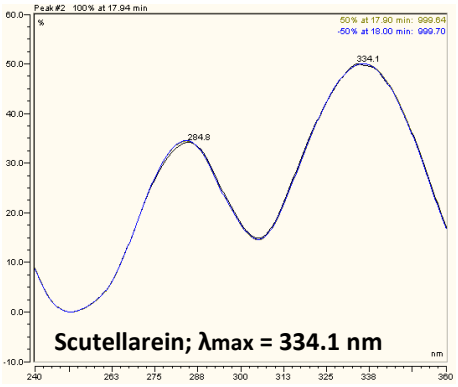


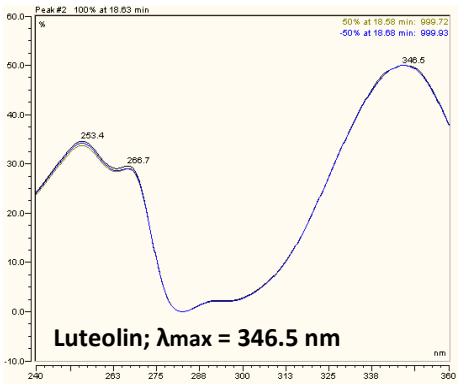
Appendix 4.2 PDA spectra of ferulic acid, rosemarinic acid, naringin, scutellarein, luteolin, and nepetin; a wavelength scanning range of 246-360 nm, 0.1 mg·mL^{-1} in methanol.

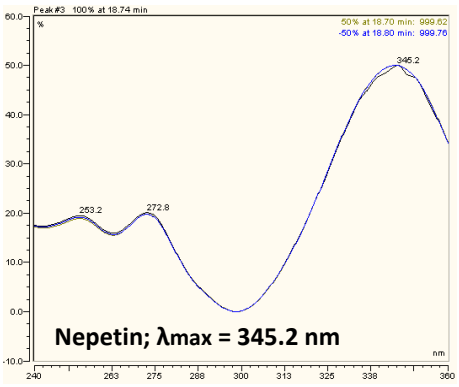




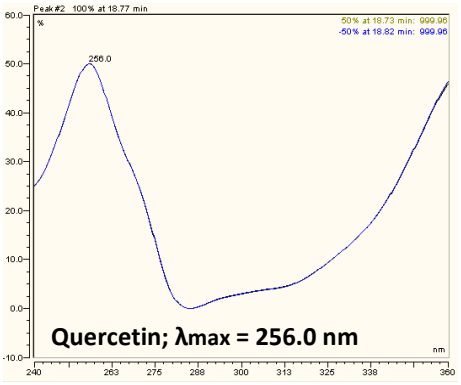


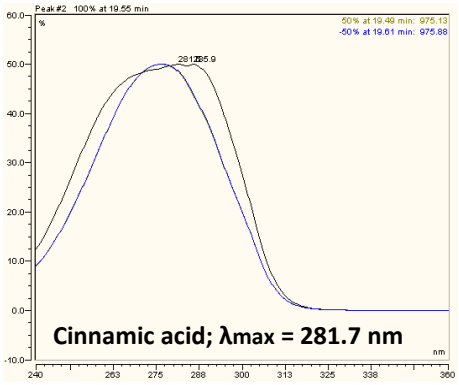


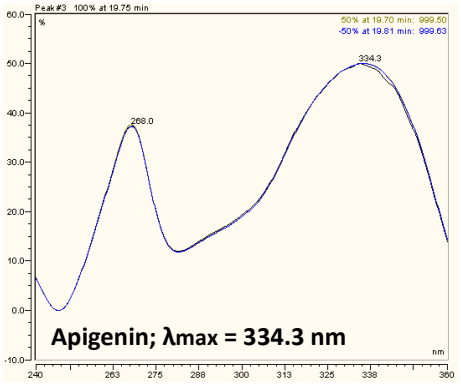


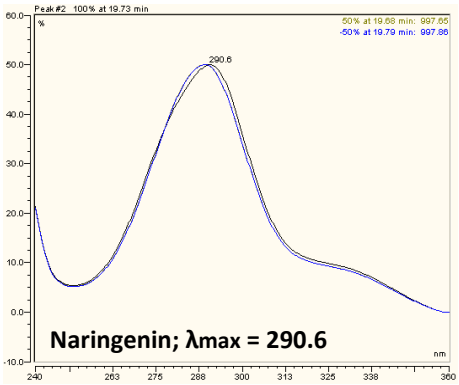


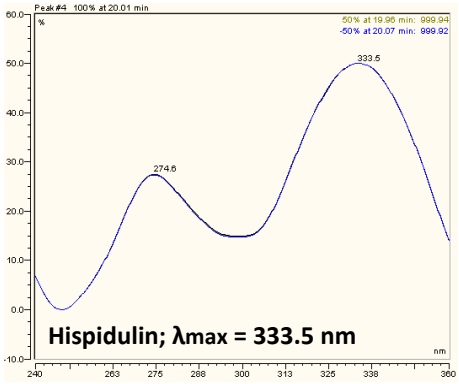
Appendix 4.3 PDA spectra of quercetin, cinnamic acid, apigenin, naringenin, hispidulin, and kaempferol; a wavelength scanning range of 246-360 nm, 0.1 mg·mL^{-1} in methanol.

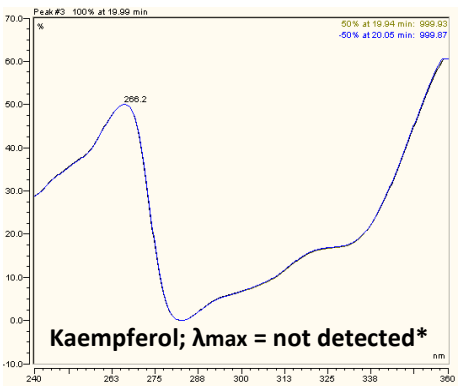






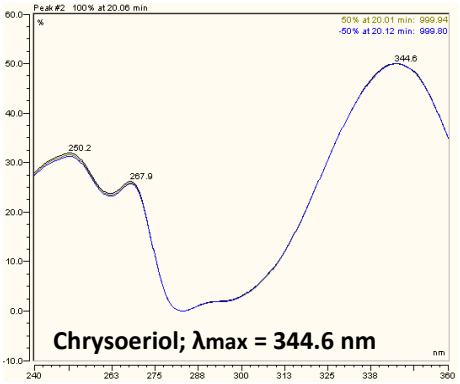


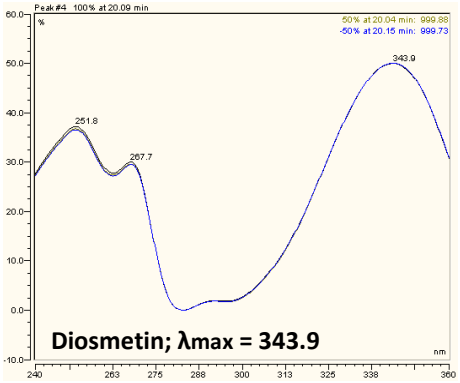


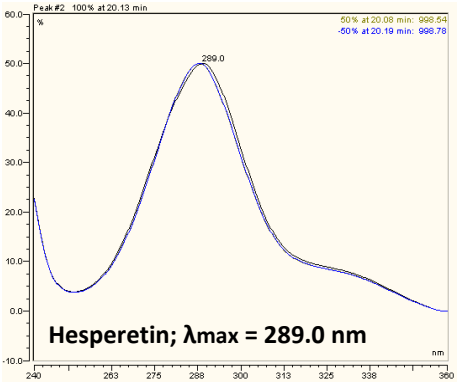


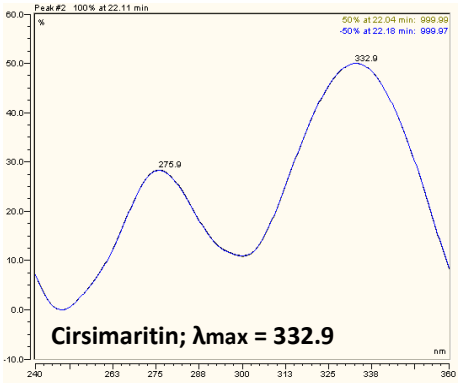
*out of scanning range

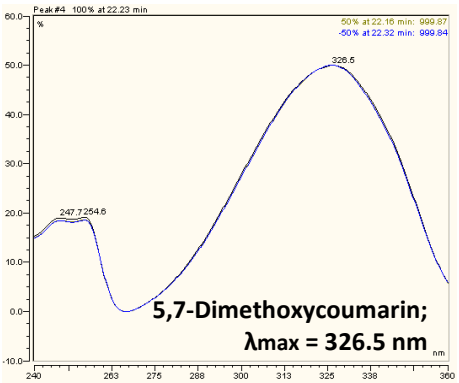
Appendix 4.4 PDA spectra of chrysoeriol, diosmetin, hesperetin, cirsimaritin, 5,7-dimethoxycoumarin, and chrysin; a wavelength scanning range of 246-360 nm, 0.1 mg·mL^{-1} in methanol.

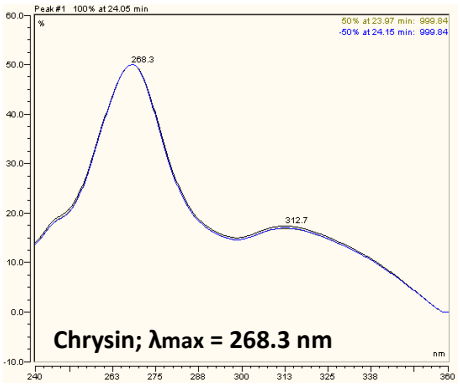






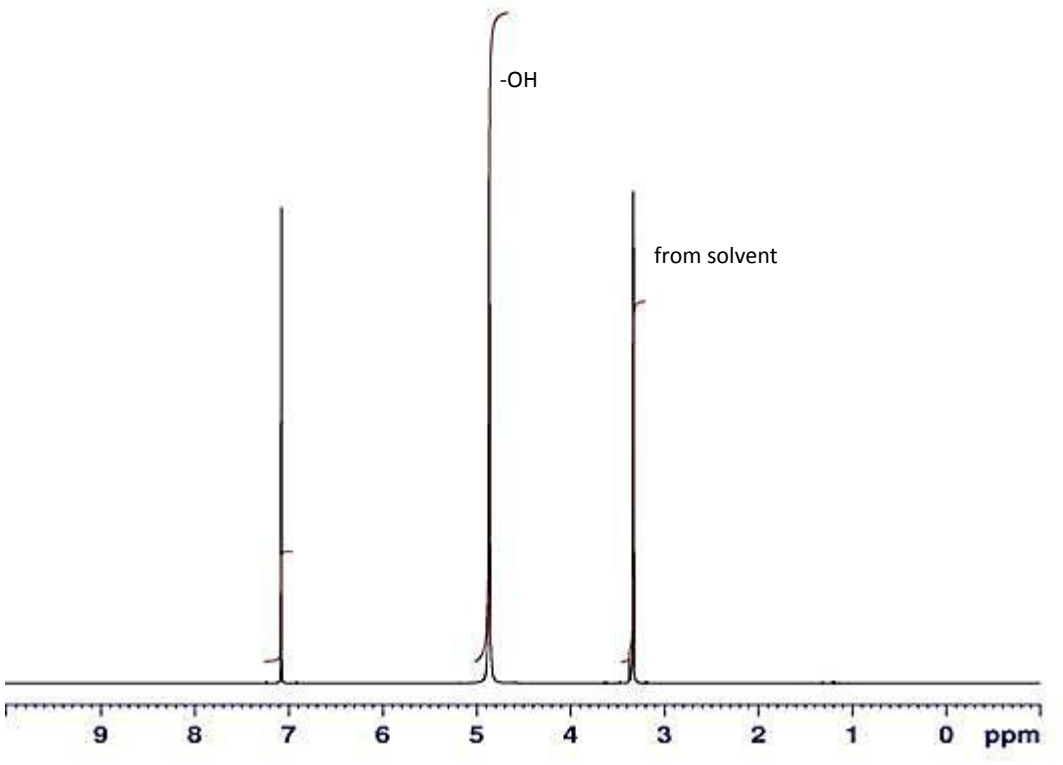




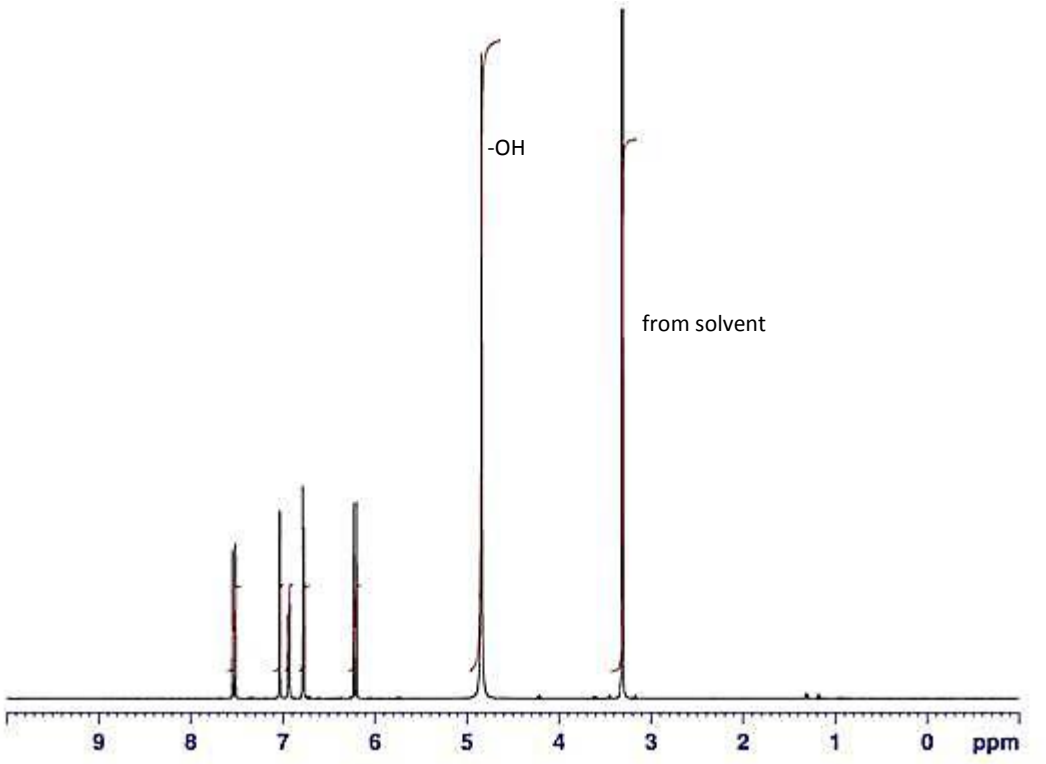


Appendix 5: NMR spectra of phenolic standards

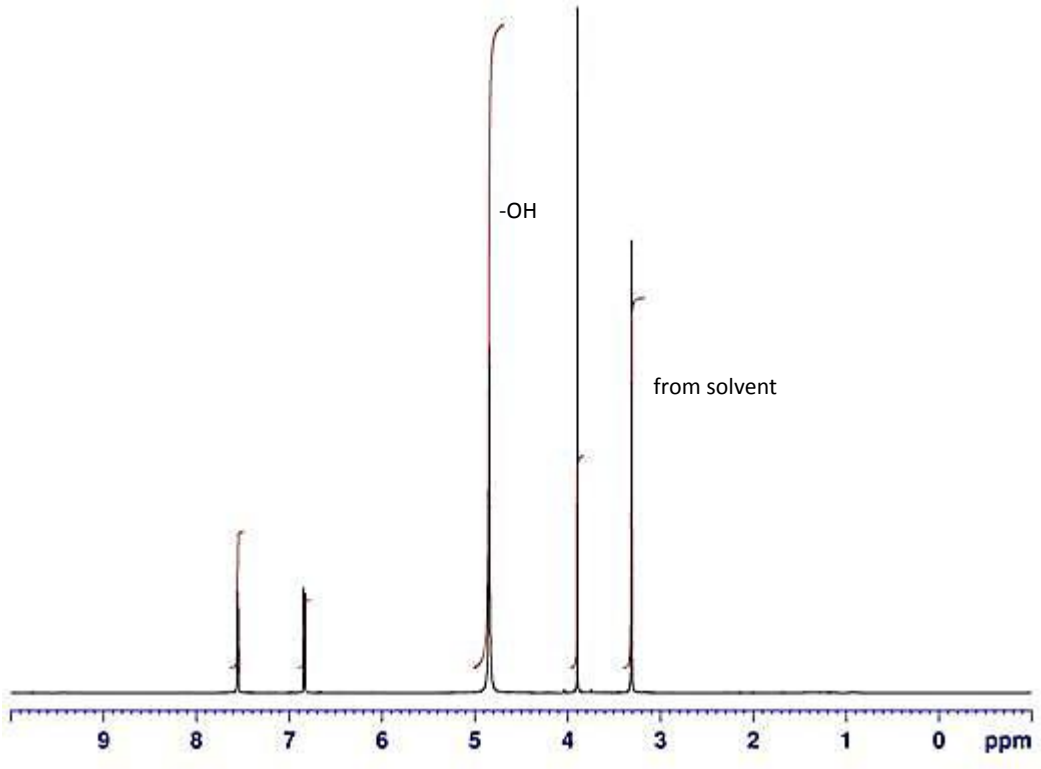
Appendix 5.1 ^{1}H NMR spectrum of gallic acid (500 MHz, CD_3OD).



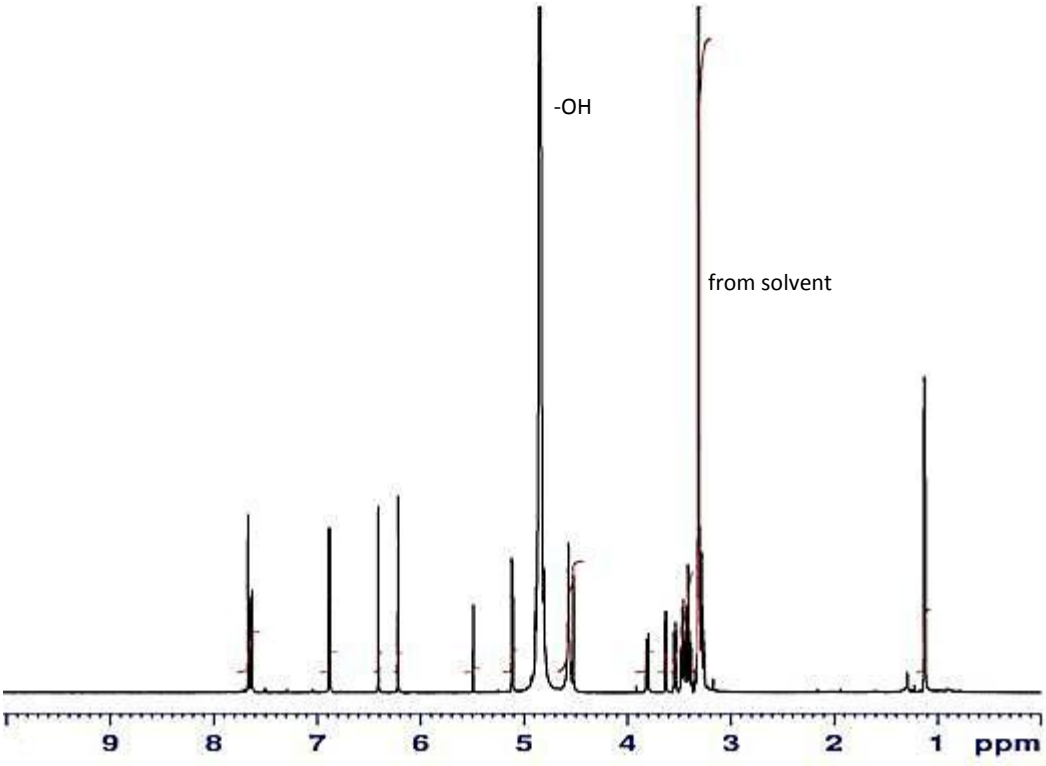
Appendix 5.2 ^{1}H NMR spectrum of caffeic acid (500 MHz, CD_3OD).



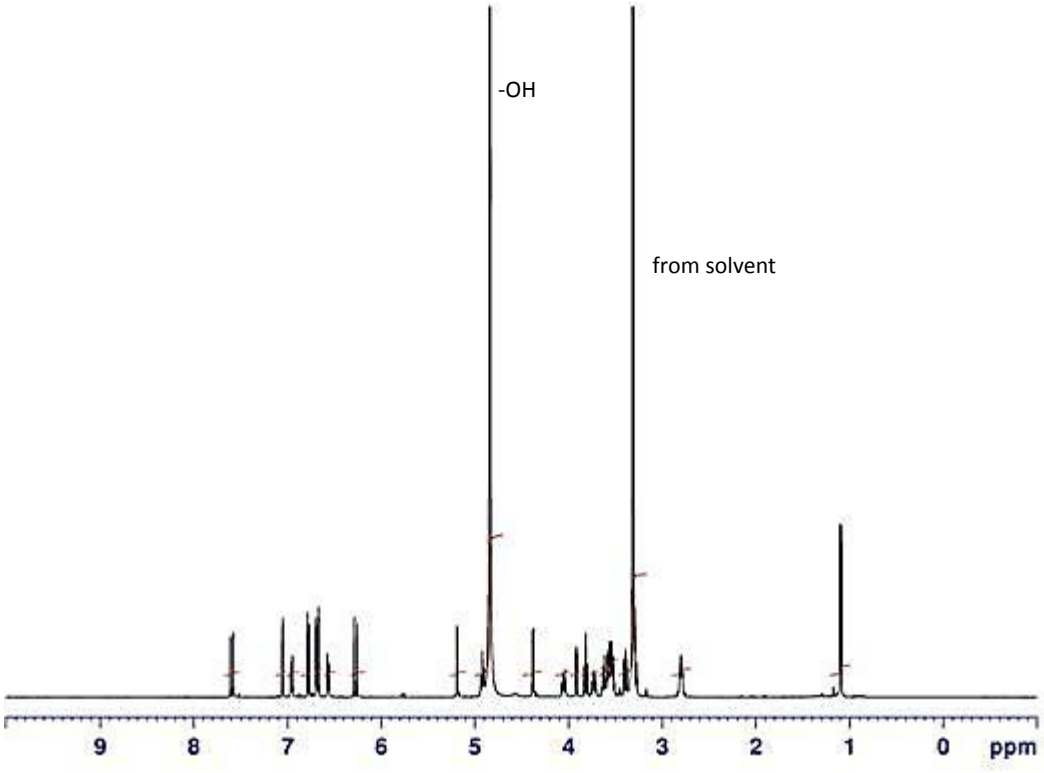
Appendix 5.3 ^{1}H NMR spectrum of vanillic acid (500 MHz, CD_3OD).



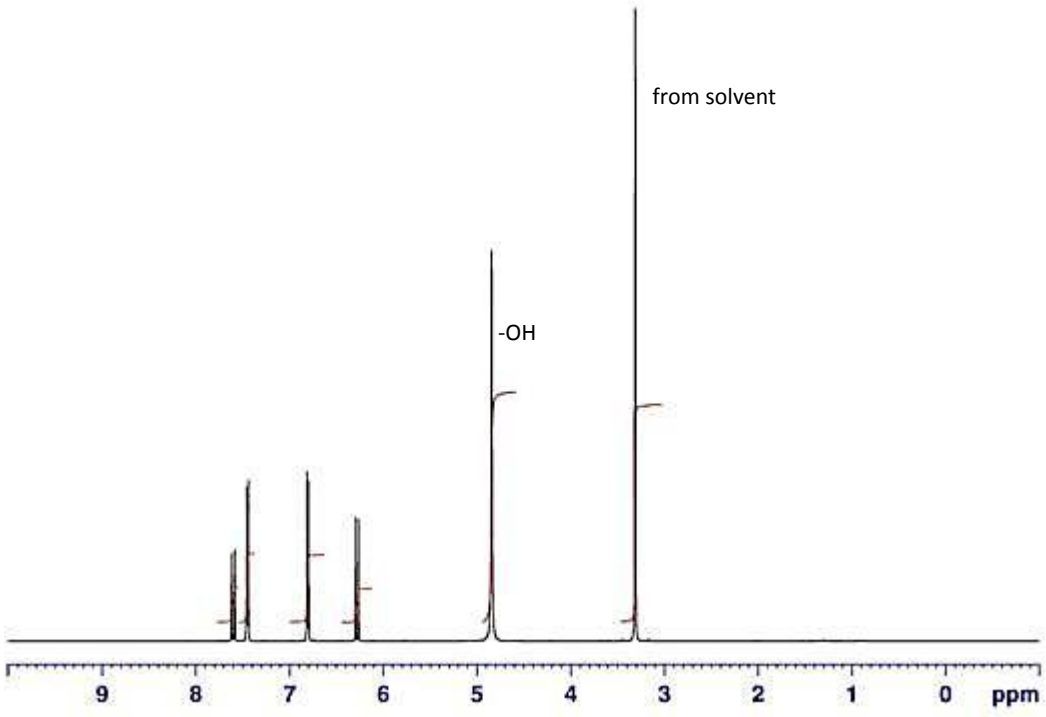
Appendix 5.4 ^{1}H NMR spectrum of rutin (500 MHz, CD_3OD).



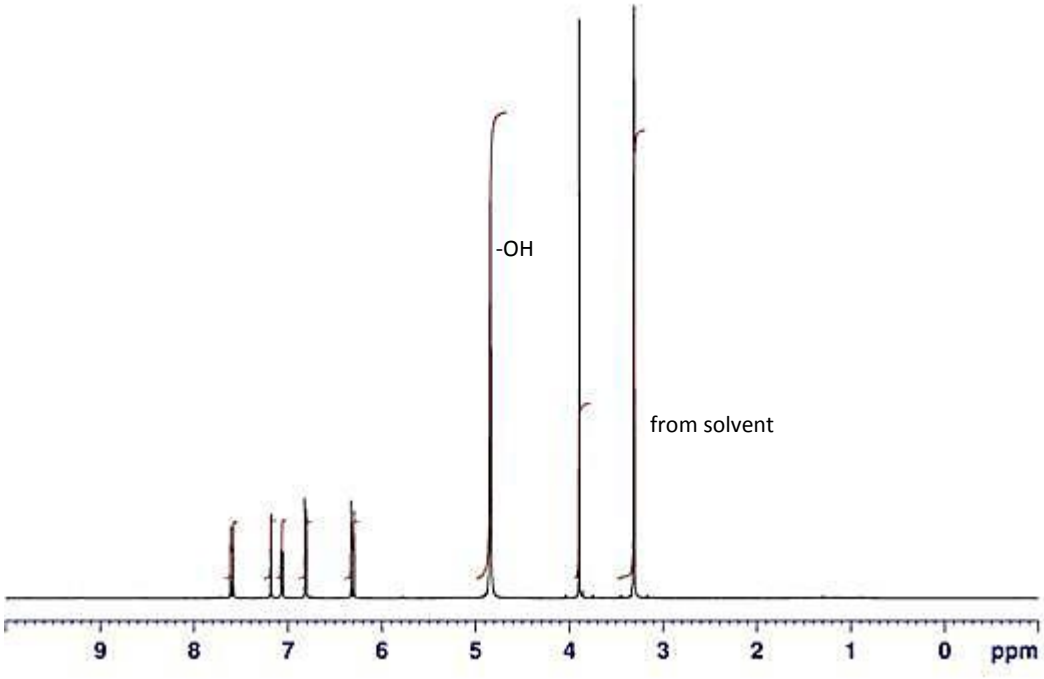
Appendix 5.5 ^{1}H NMR spectrum of verbascoside (500 MHz, CD_3OD).



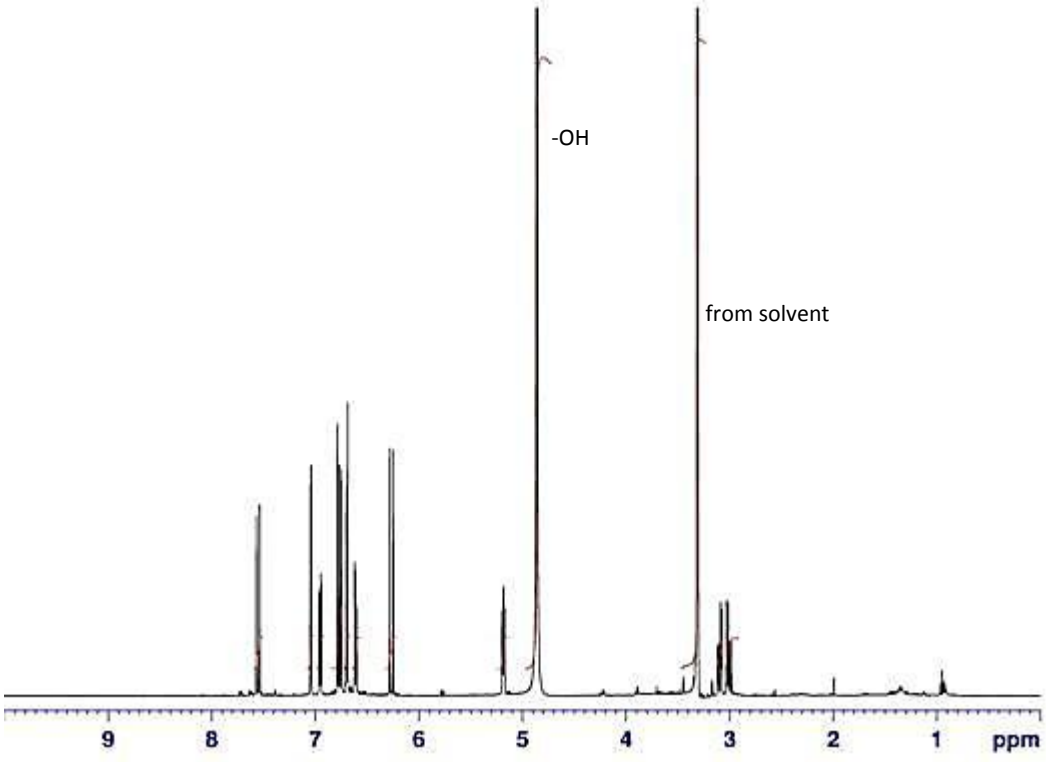
Appendix 5.6 ^{1}H NMR spectrum of 4-coumaric acid (500 MHz, CD_3OD).



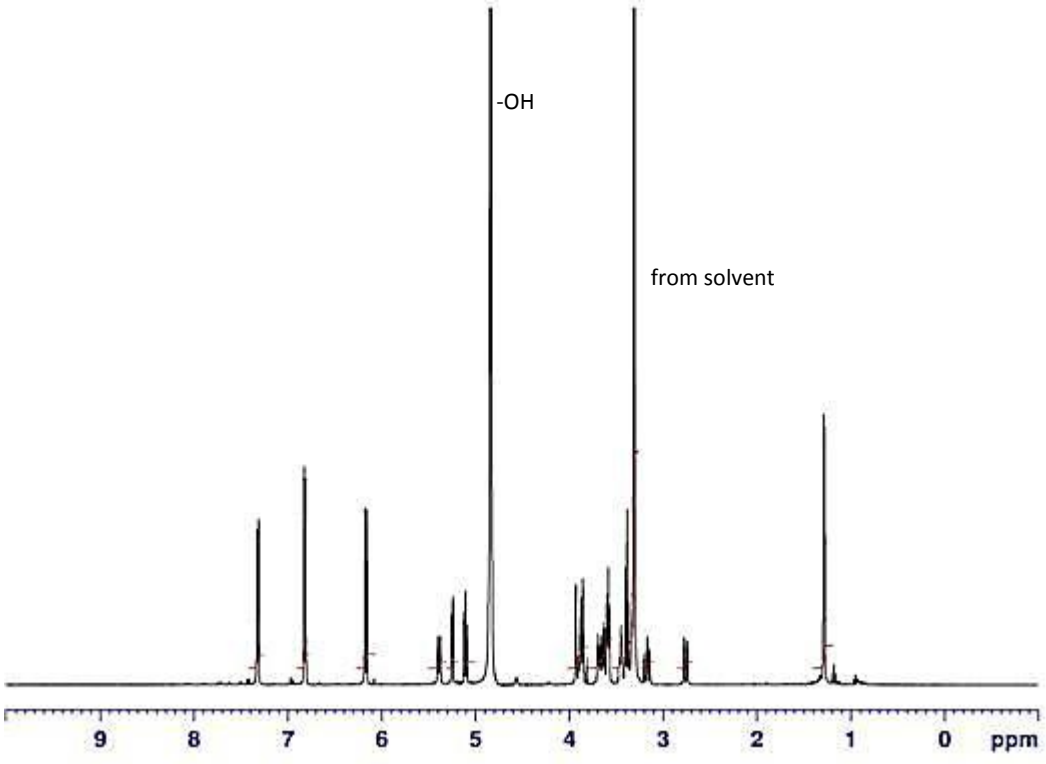
Appendix 5.7 ^{1}H NMR spectrum of ferulic acid (500 MHz, CD_3OD).



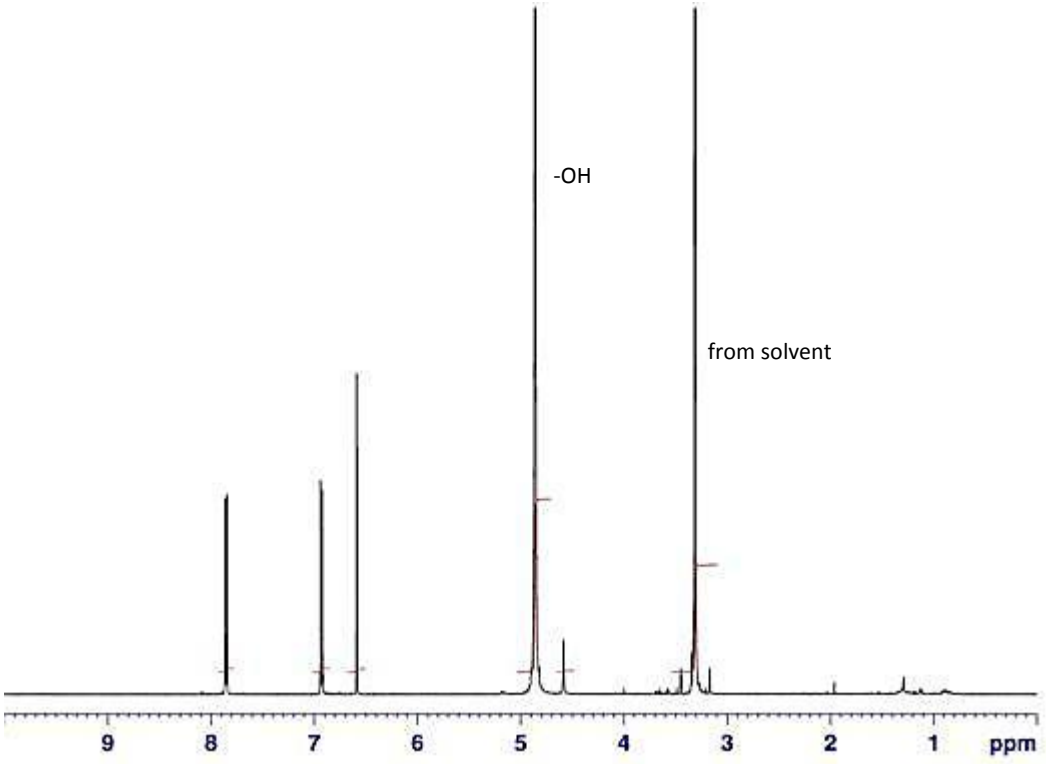
Appendix 5.8 ^{1}H NMR spectrum of rosemarinic acid (500 MHz, CD_3OD).



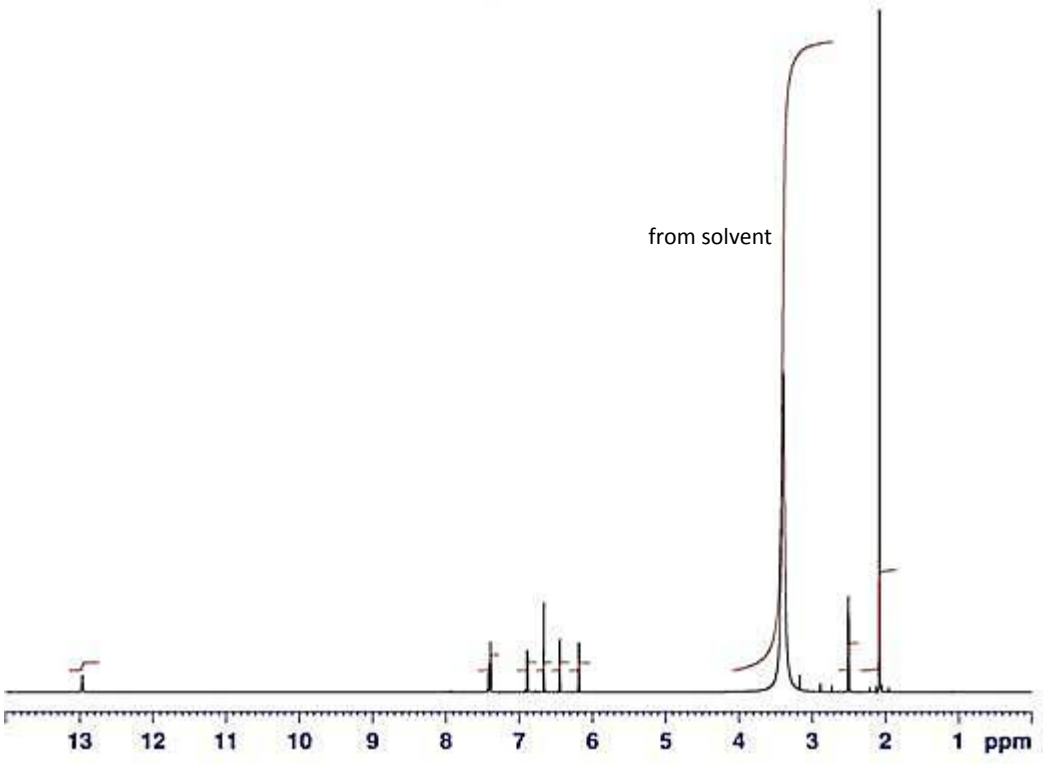
Appendix 5.9 ^{1}H NMR spectrum of naringin (500 MHz, CD_3OD).



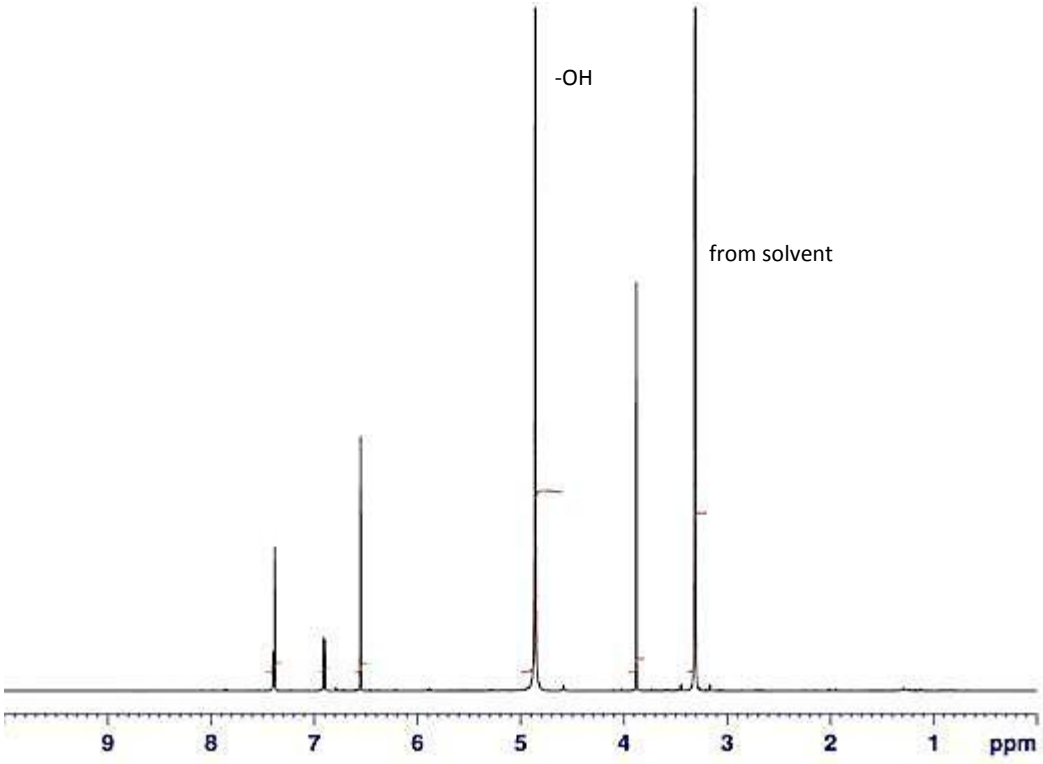
Appendix 5.10 ^{1}H NMR spectrum of scutellarein (500 MHz, CD_3OD).



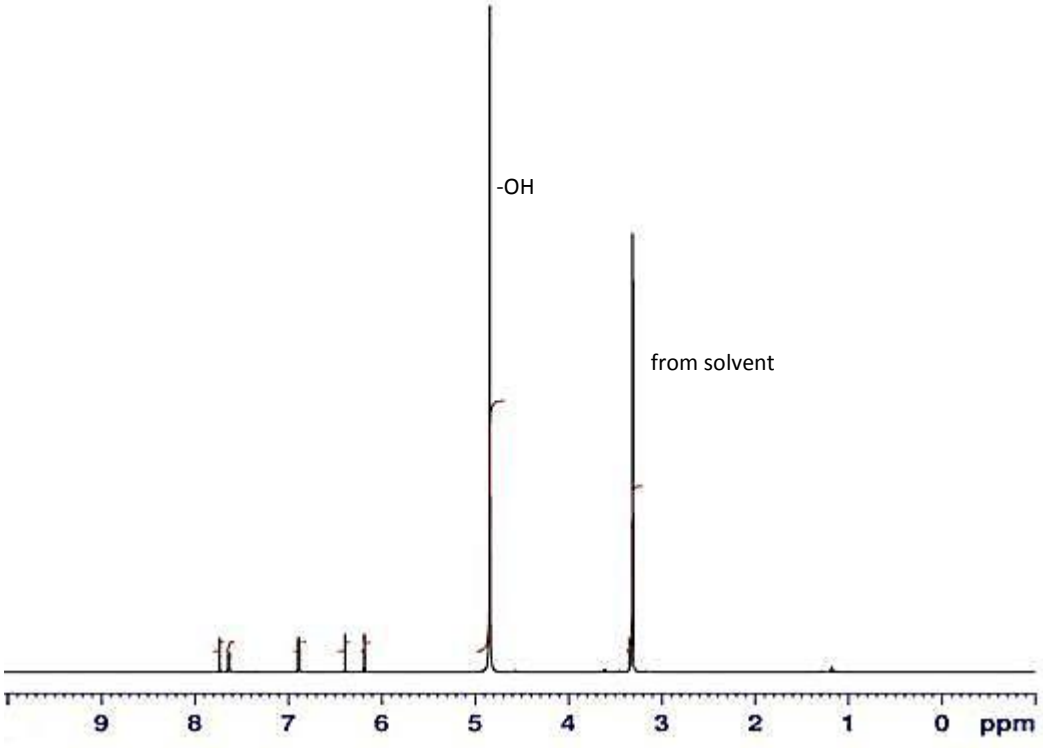
Appendix 5.11 ^{1}H NMR spectrum of luteolin (500 MHz, CD_3OD).



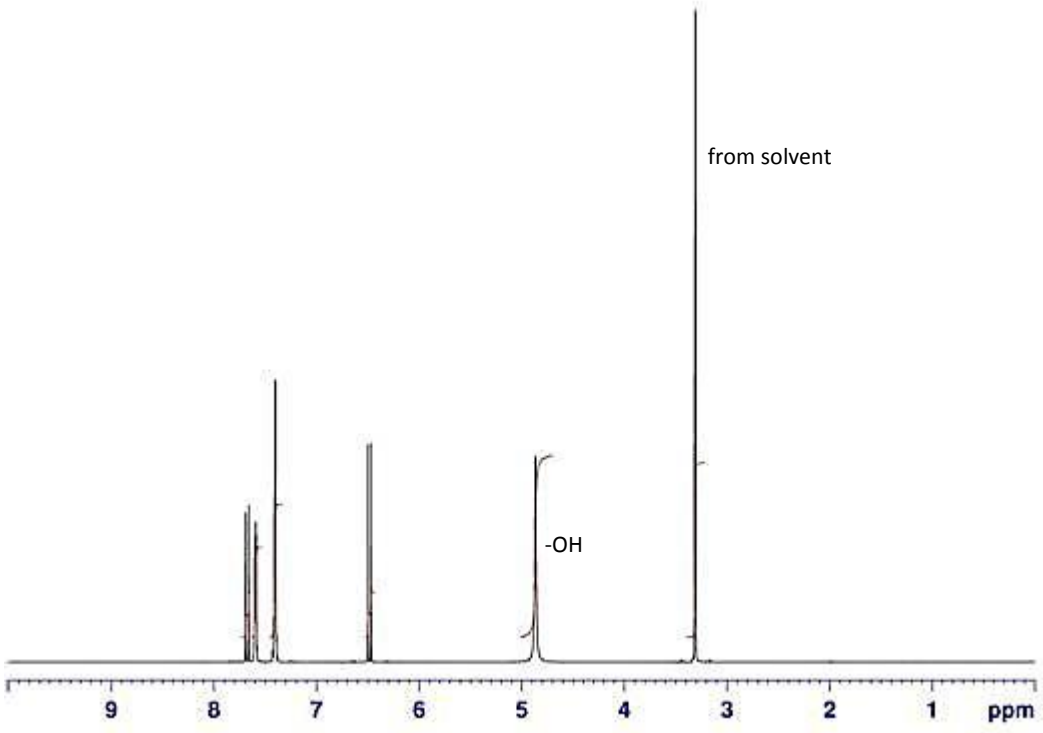
Appendix 5.12 ^{1}H NMR spectrum of nepetin (500 MHz, CD_3OD).



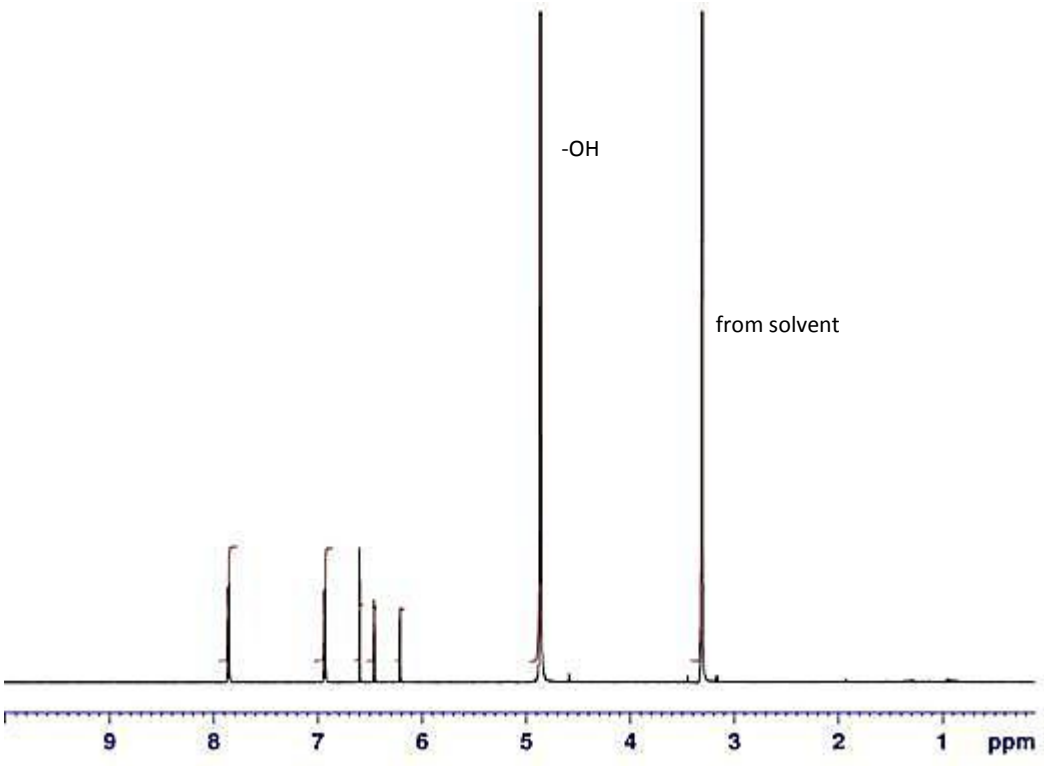
Appendix 5.13 ^{1}H NMR spectrum of quercetin (500 MHz, CD_3OD).



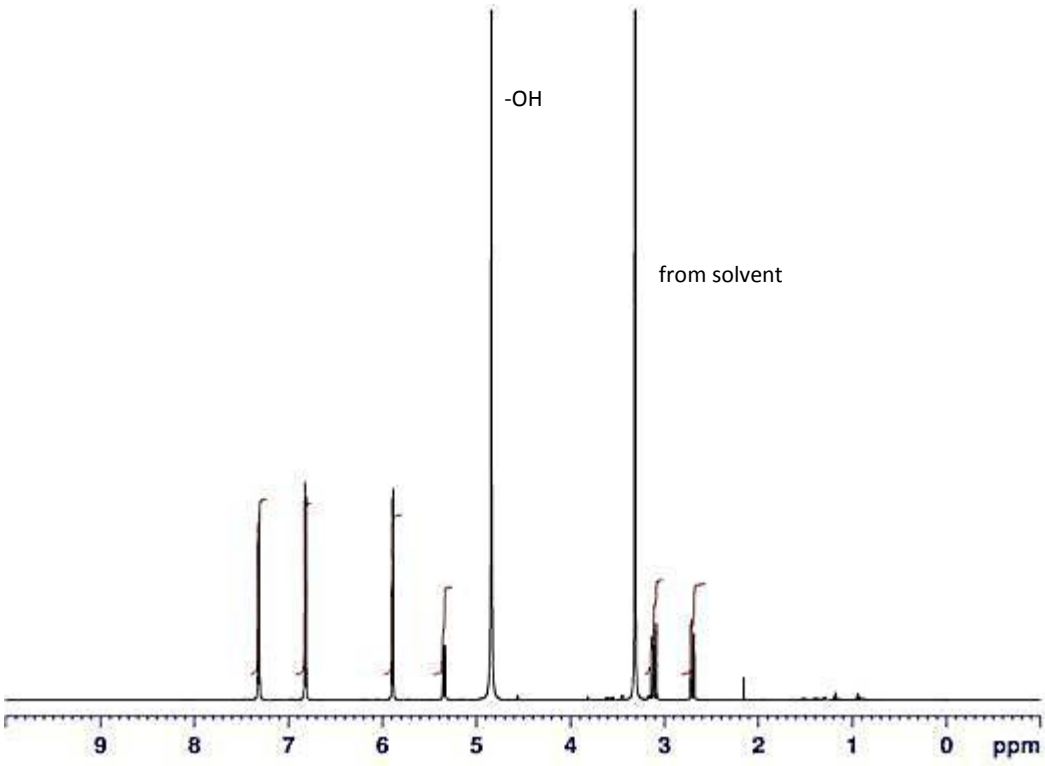
Appendix 5.14 ^{1}H NMR spectrum of cinnamic acid (500 MHz, CD_3OD).



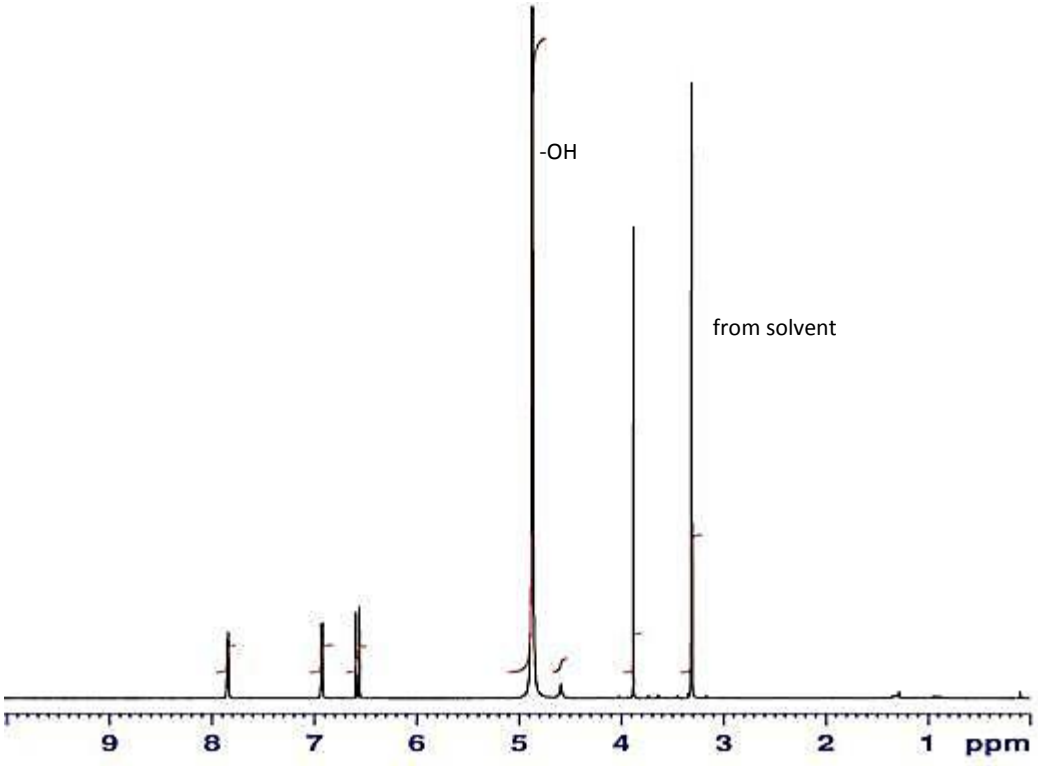
Appendix 5.15 ^{1}H NMR spectrum of apigenin (500 MHz, CD_3OD).



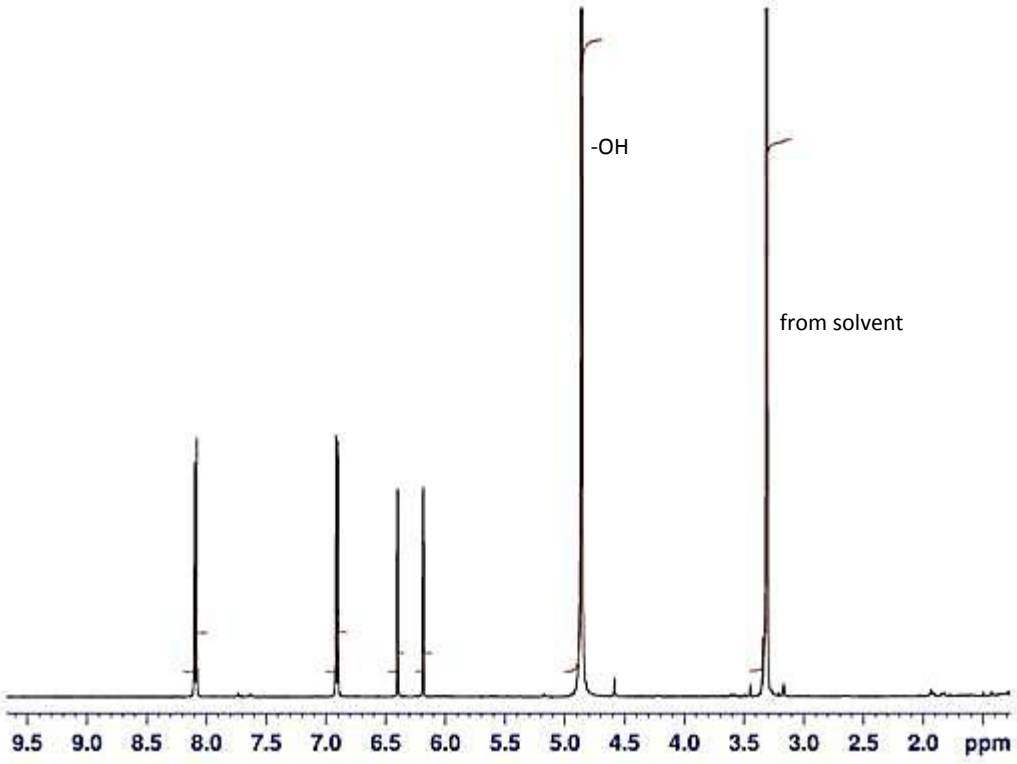
Appendix 5.16 ^{1}H NMR spectrum of naringenin (500 MHz, CD_3OD).



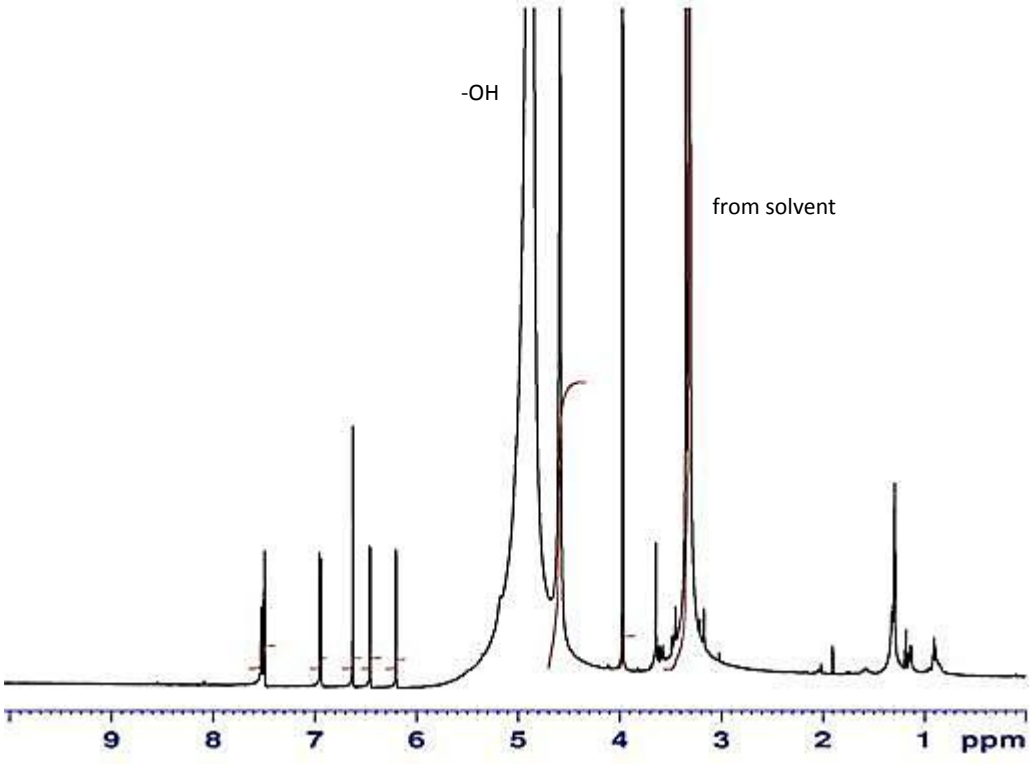
Appendix 5.17 ^{1}H NMR spectrum of hsipidulin (500 MHz, CD_3OD).



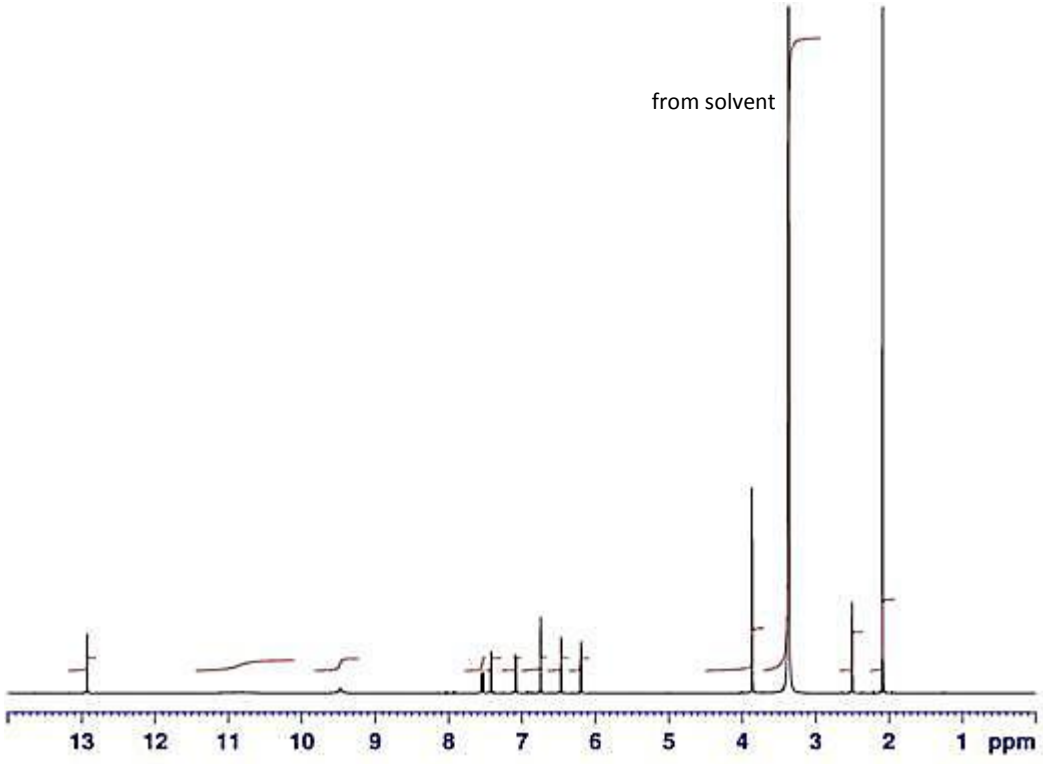
Appendix 5.18 ^{1}H NMR spectrum of kaempferol (500 MHz, CD_3OD).



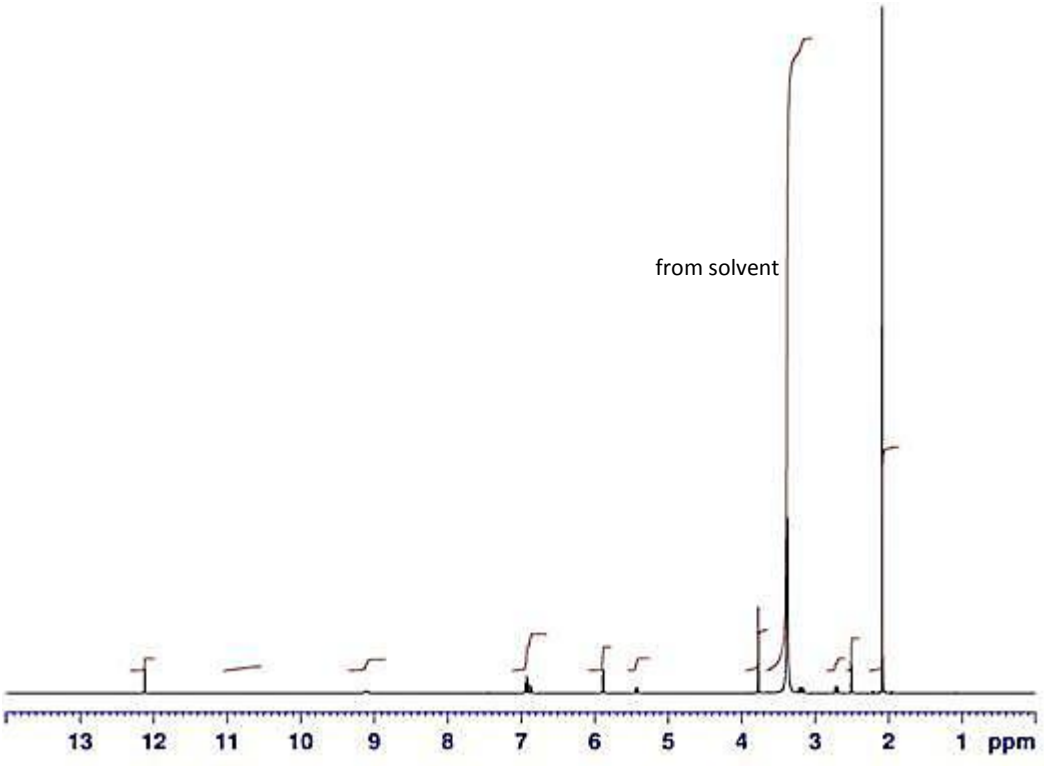
Appendix 5.19 ^{1}H NMR spectrum of chrysoeriol (500 MHz, CD_3OD).



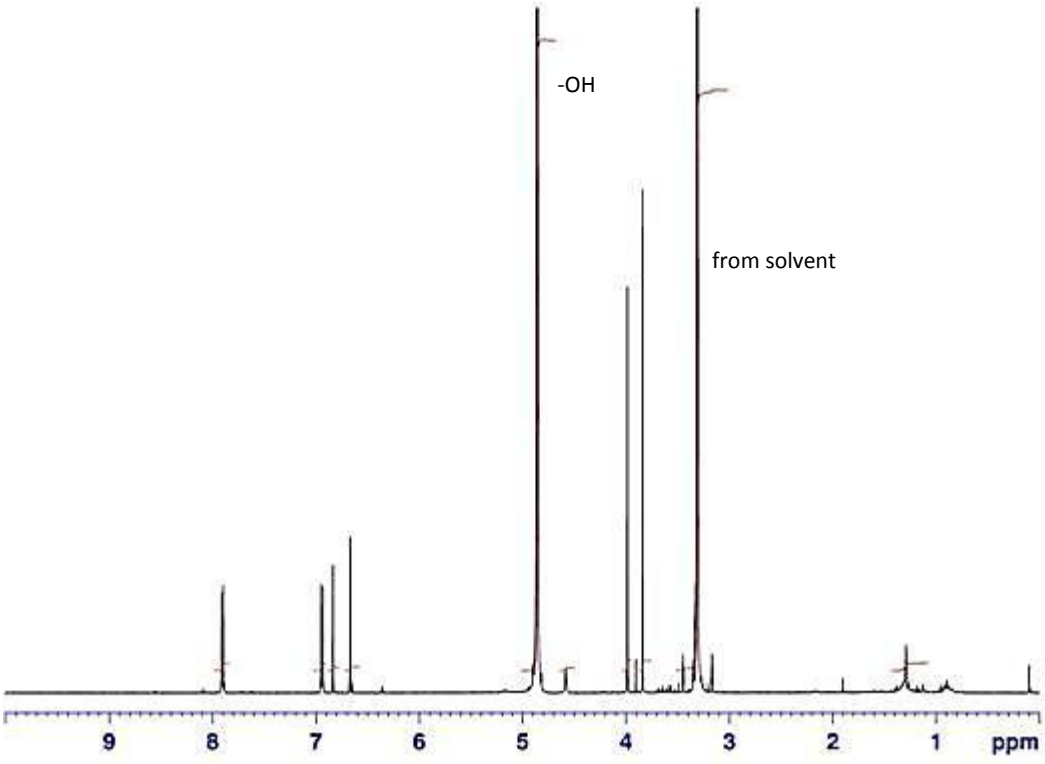
Appendix 5.20 ^{1}H NMR spectrum of diosmetin (500 MHz, CD_3OD).



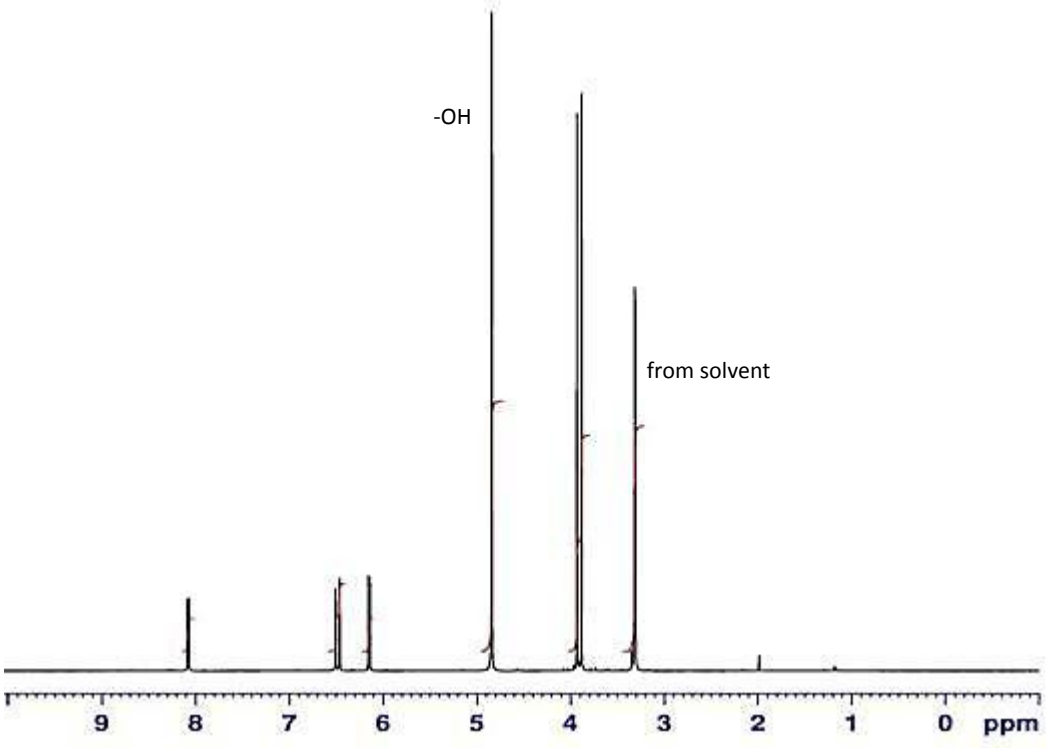
Appendix 5.21 ^{1}H NMR spectrum of hesperetin (500 MHz, CD_3OD).



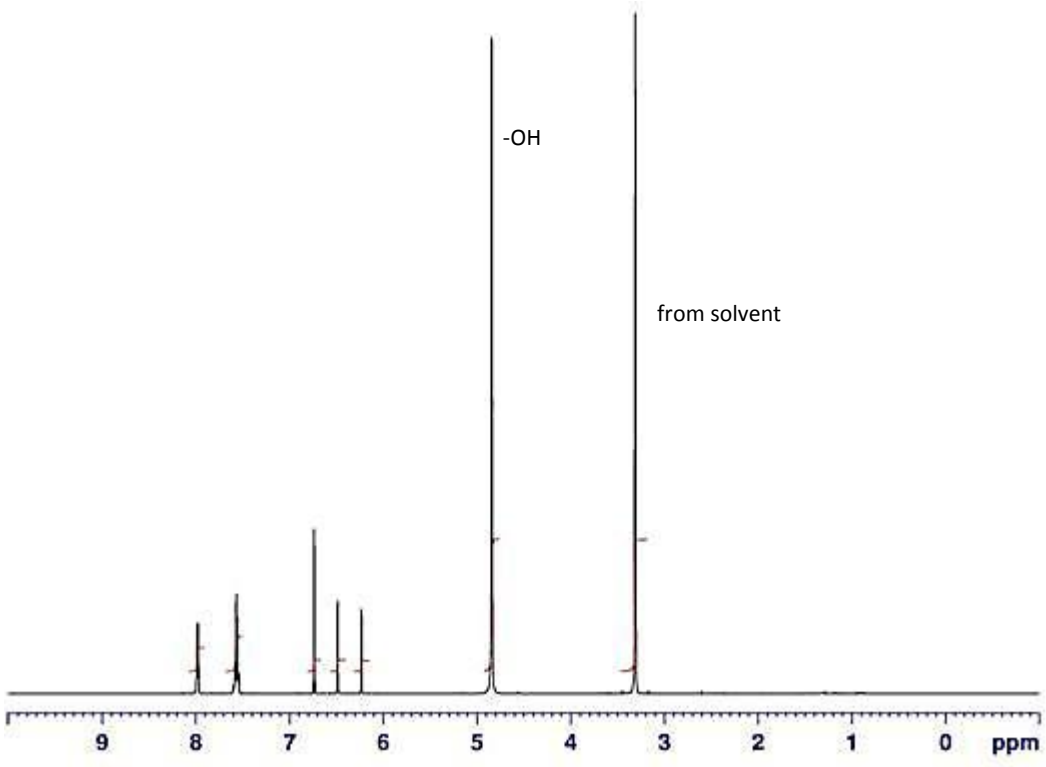
Appendix 5.22 ^{1}H NMR spectrum of cirsimaritin (500 MHz, CD_3OD).



Appendix 5.23 ^{1}H NMR spectrum of 5,7-dimethoxycoumarin (500 MHz, CD_3OD).

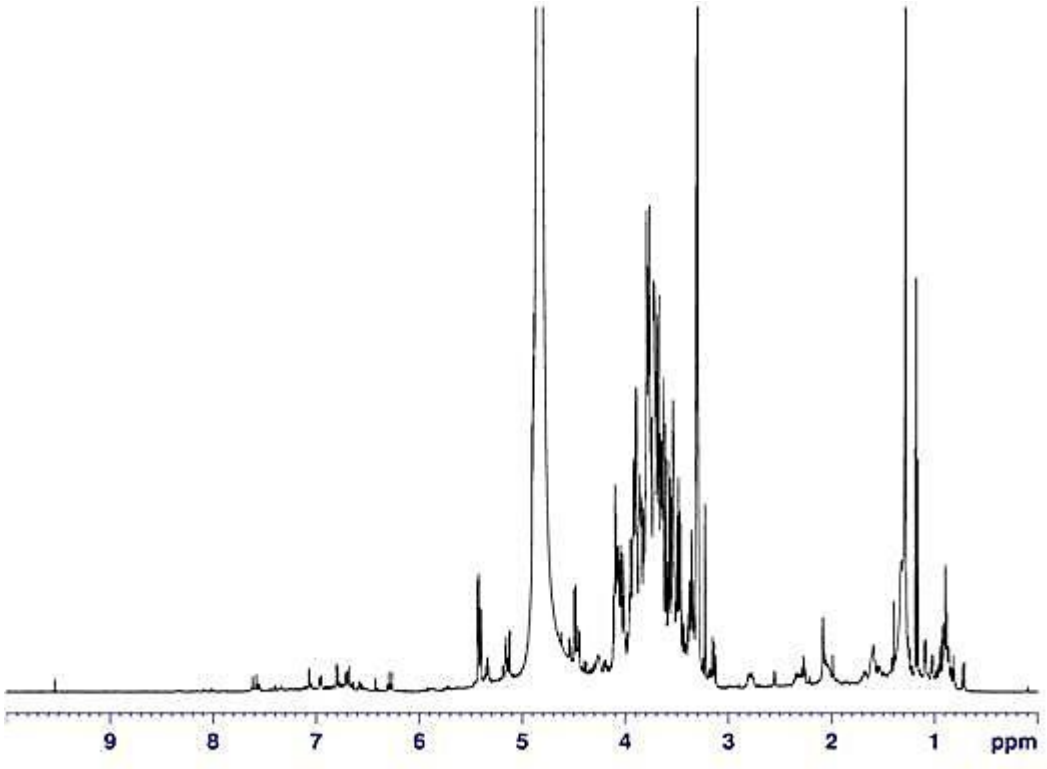


Appendix 5.24 ^{1}H NMR spectrum of chrysin (500 MHz, CD_3OD).

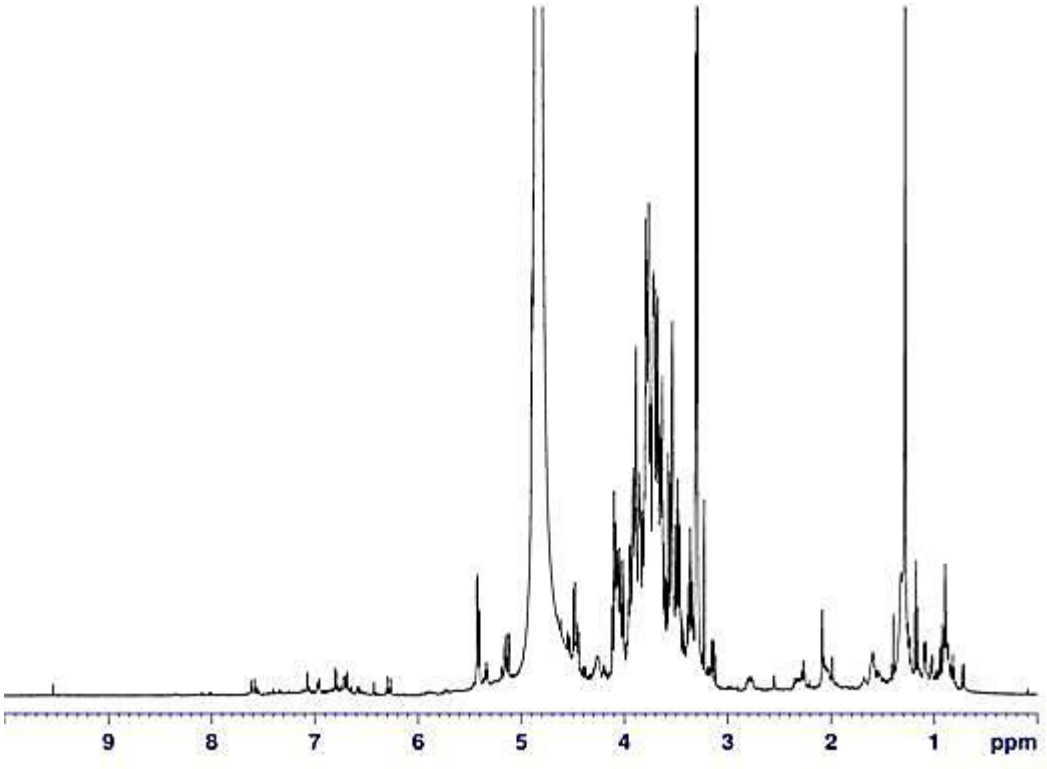


Appendix 6: NMR spectra of *A. ebracteatus* in five batches of ethanolic extracts

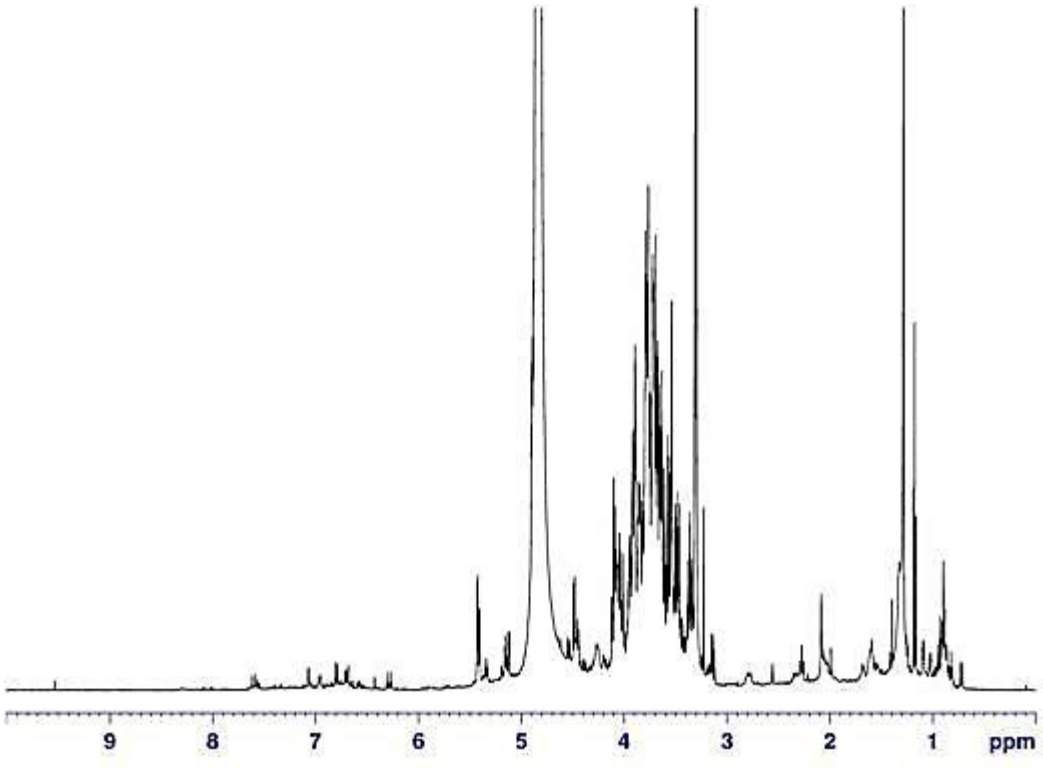
Appendix 6.1 ^{1}H NMR spectrum of *A. ebracteatus* in batch 1 (500 MHz, CD_3OD).



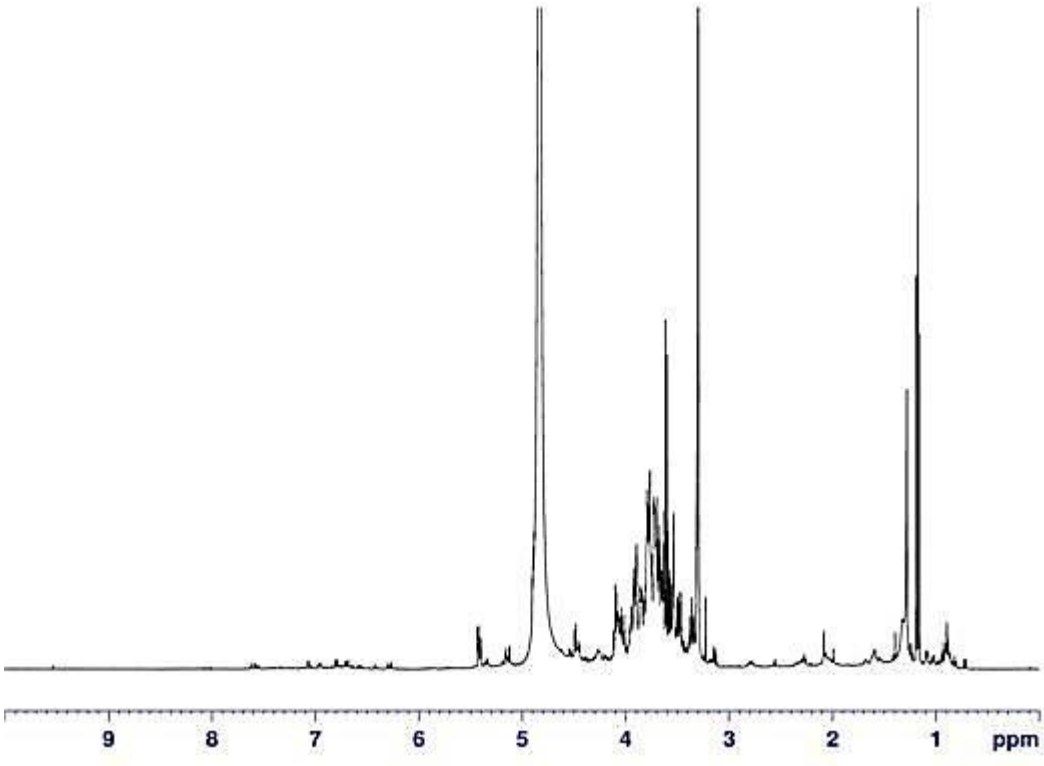
Appendix 6.2 ^{1}H NMR spectrum of *A. ebracteatus* in batch 2 (500 MHz, CD_3OD).



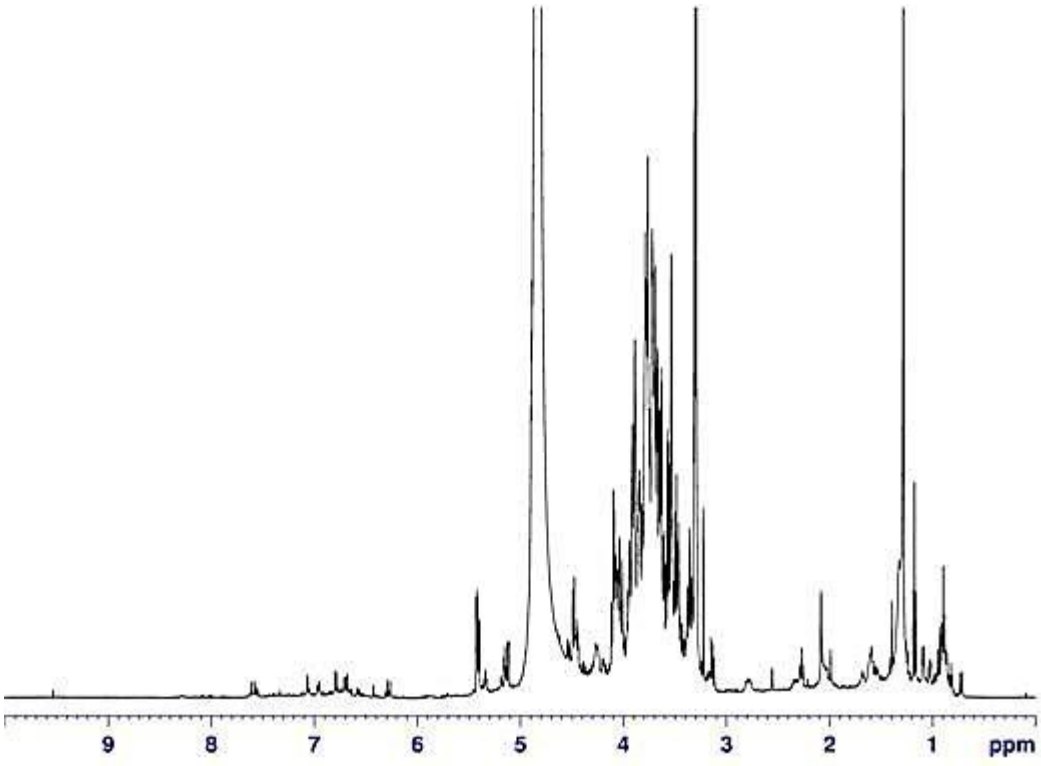
Appendix 6.3 ^{1}H NMR spectrum of *A. ebracteatus* in batch 3 (500 MHz, CD_3OD).



Appendix 6.4 ^{1}H NMR spectrum of *A. ebracteatus* in batch 4 (500 MHz, CD_3OD).

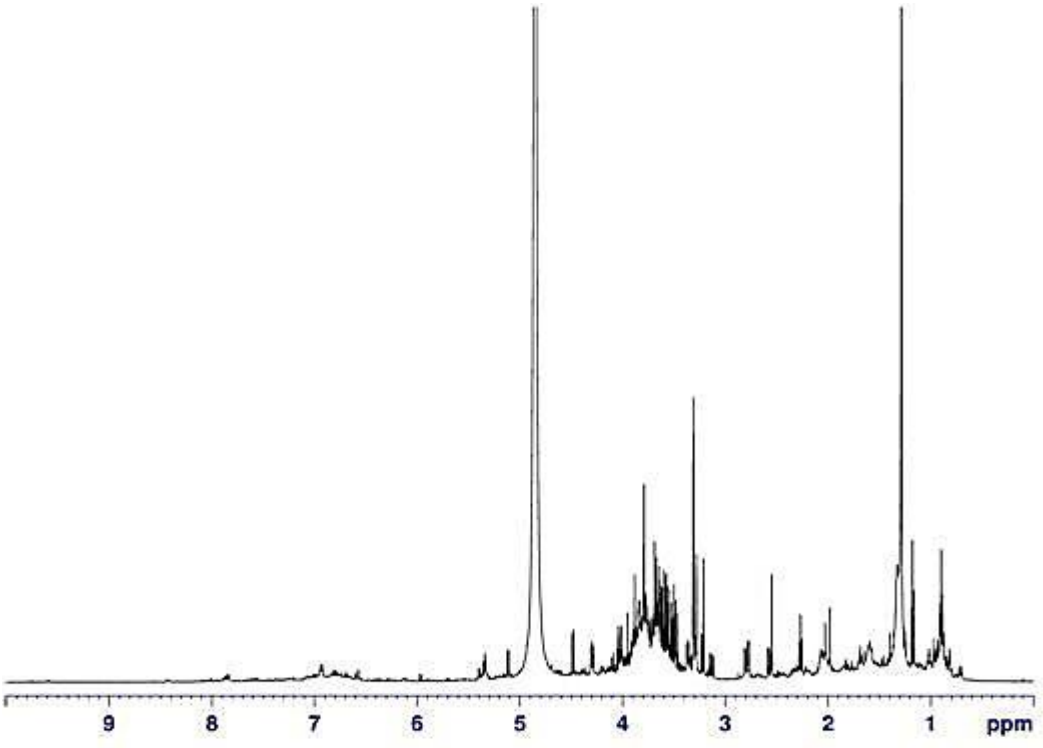


Appendix 6.5 ^{1}H NMR spectrum of *A. ebracteatus* in batch 5 (500 MHz, CD_3OD).

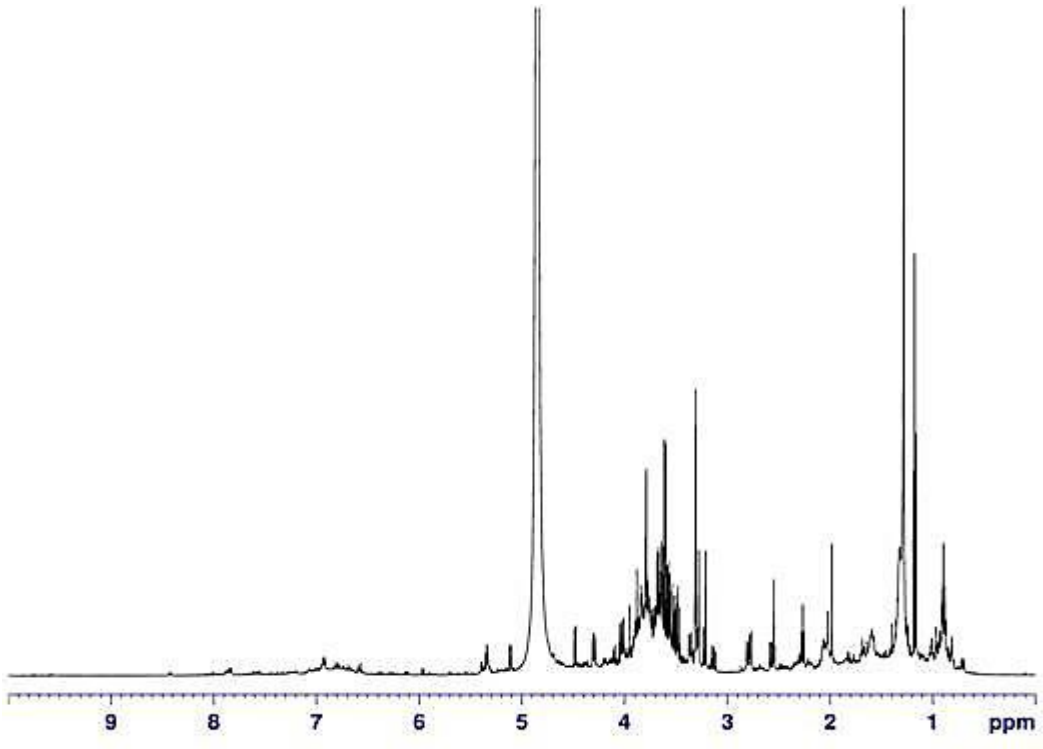


Appendix 7: NMR spectra of *C. petasites* in five batches of ethanolic extracts and one batch of SFE extract

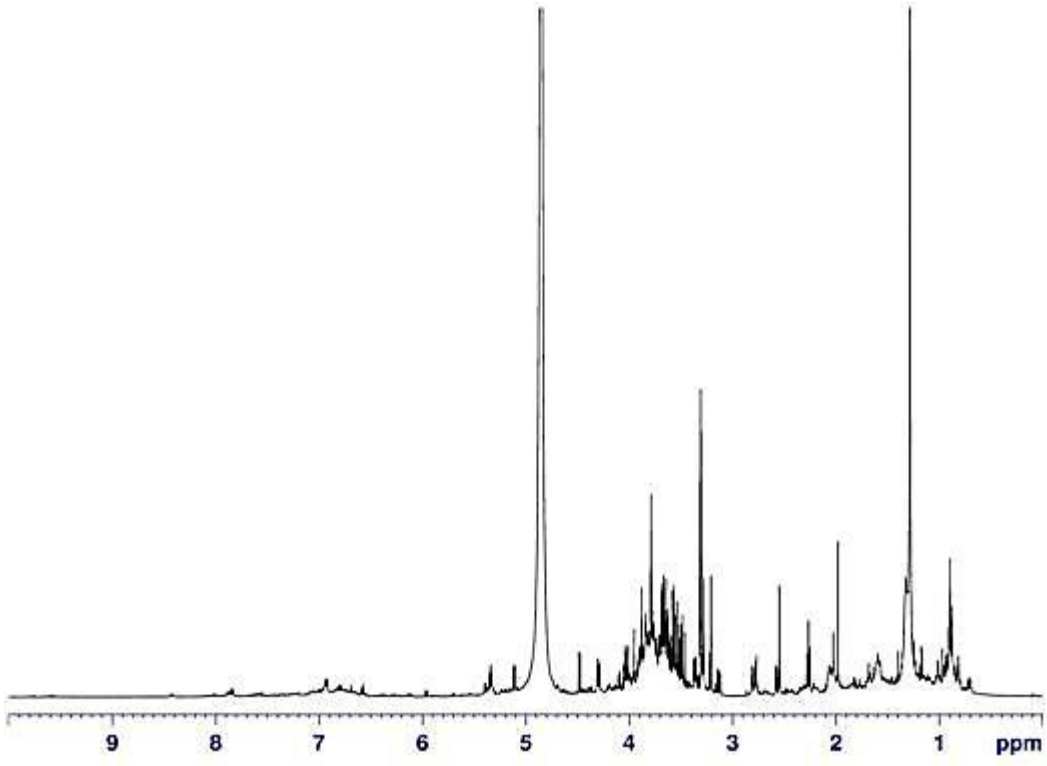
Appendix 7.1 ^{1}H NMR spectrum of *C. petasites* in batch 1 (500 MHz, CD_3OD).



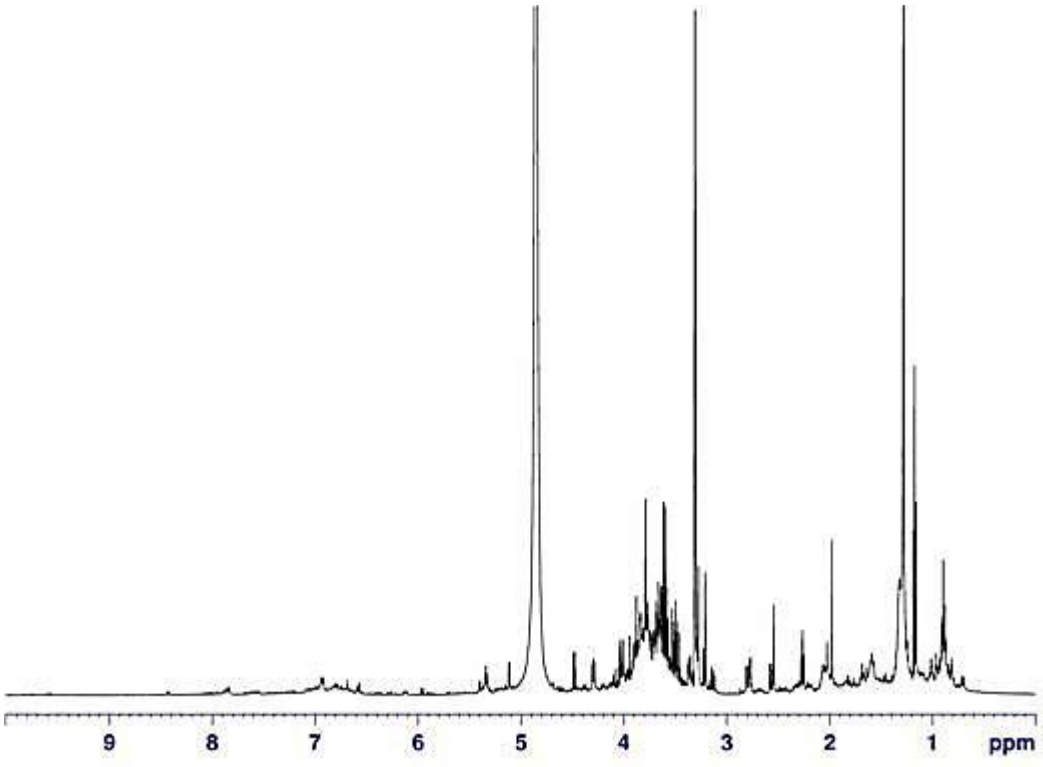
Appendix 7.2 ^{1}H NMR spectrum of *C. petasites* in batch 2 (500 MHz, CD_3OD).



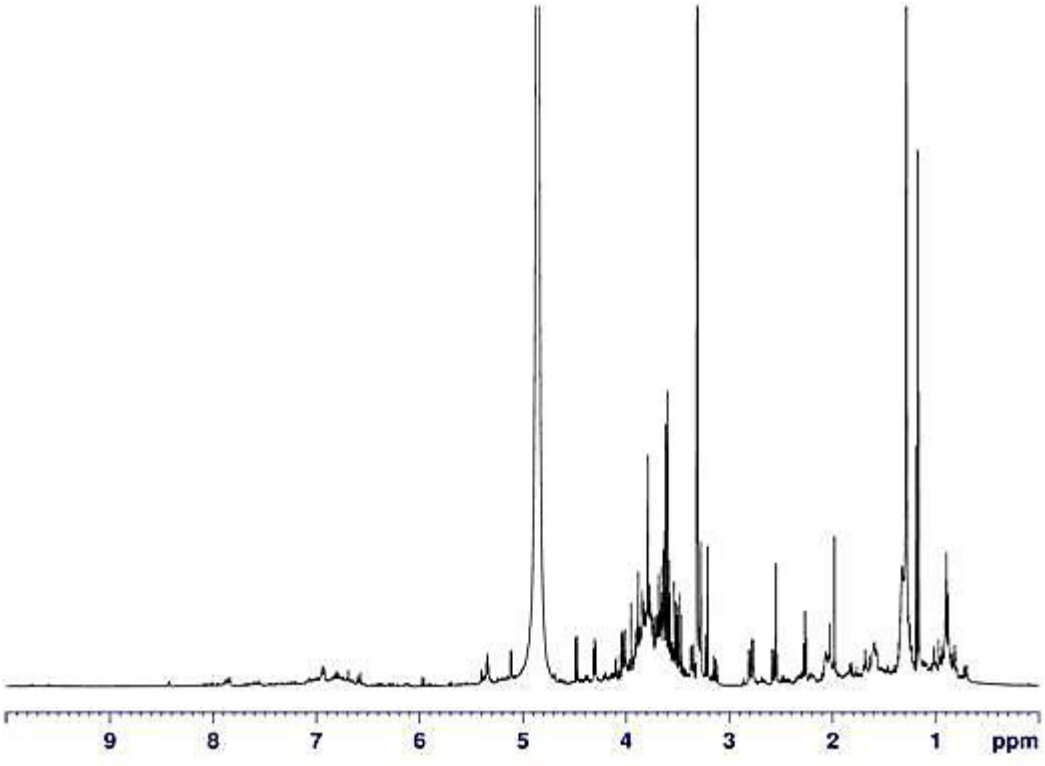
Appendix 7.3 ^{1}H NMR spectrum of *C. petasites* in batch 3 (500 MHz, CD_3OD).



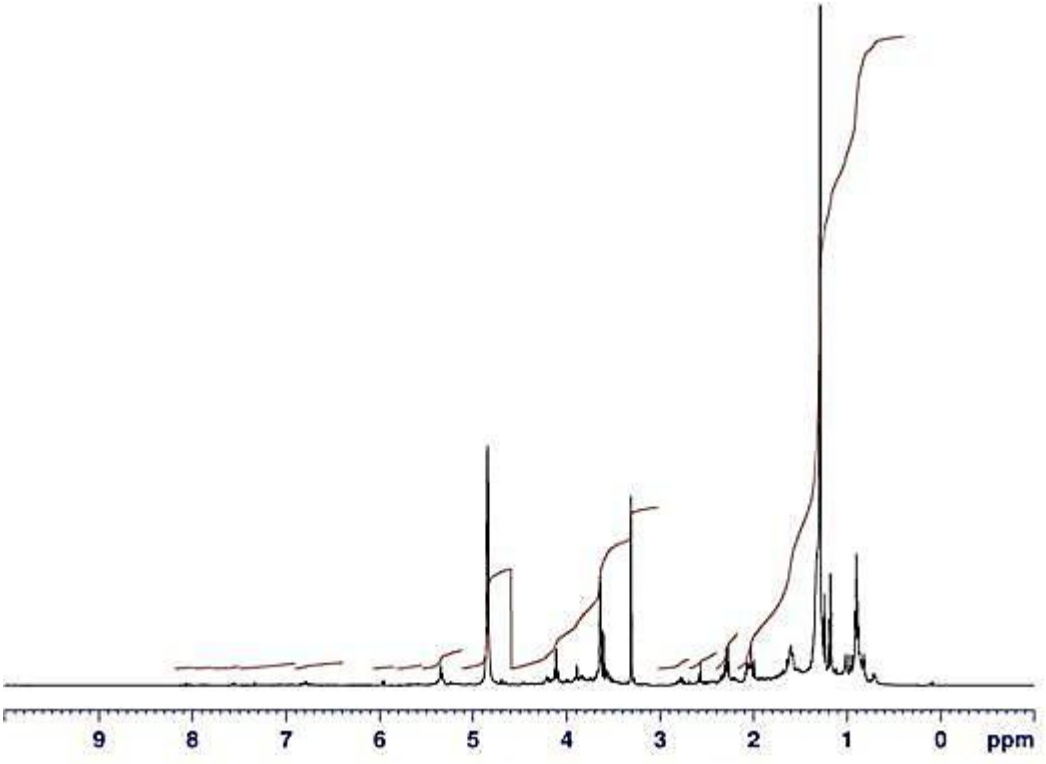
Appendix 7.4 ^{1}H NMR spectrum of *C. petasites* in batch 4 (500 MHz, CD_3OD).



Appendix 7.5 ^{1}H NMR spectrum of *C. petasites* in batch 5 (500 MHz, CD_3OD).

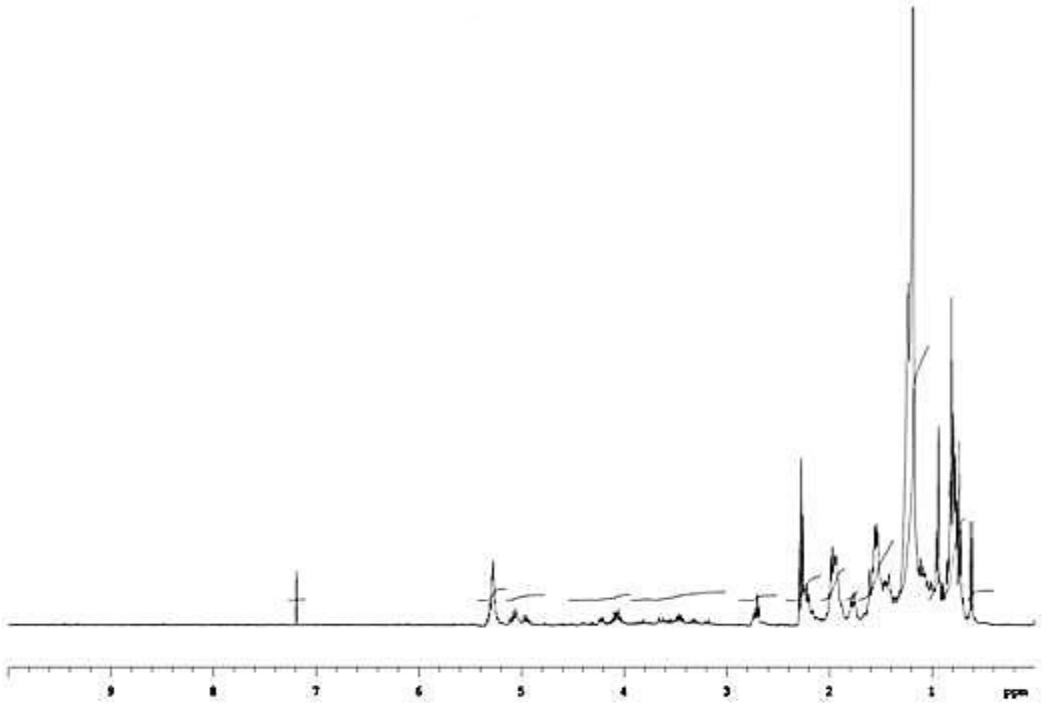


Appendix 7.6 ^{1}H NMR spectrum of *C. petasites* in SFE extract (500 MHz, CD_3OD).

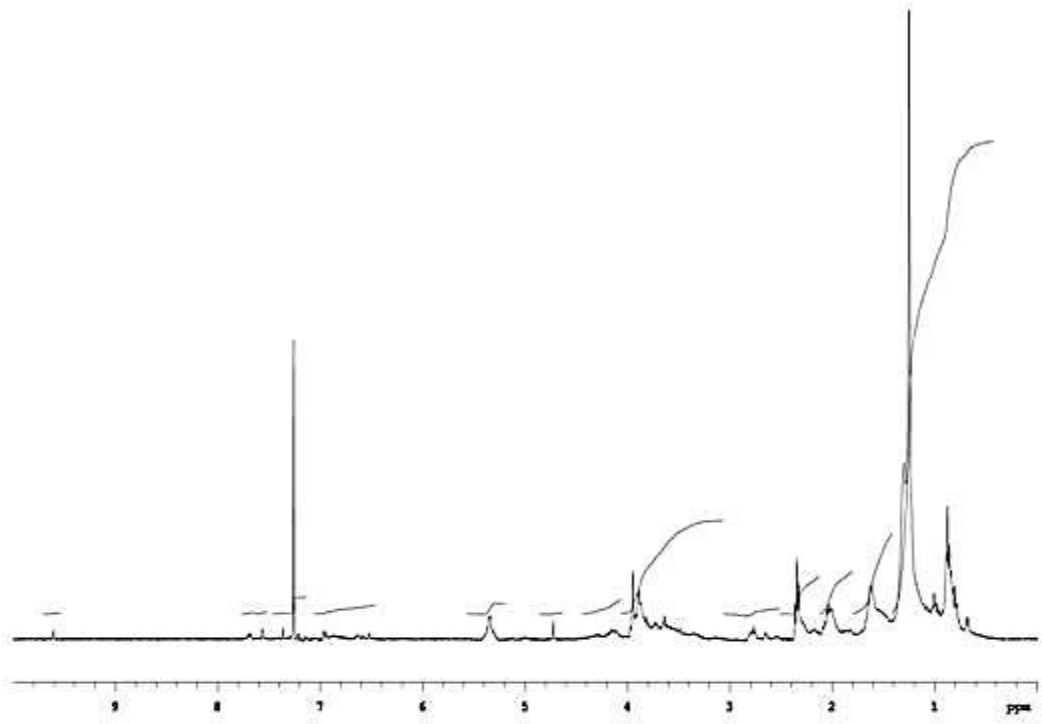


Appendix 8: NMR spectra of *A. ebracteatus* in four fractions separated by liquid-liquid partition

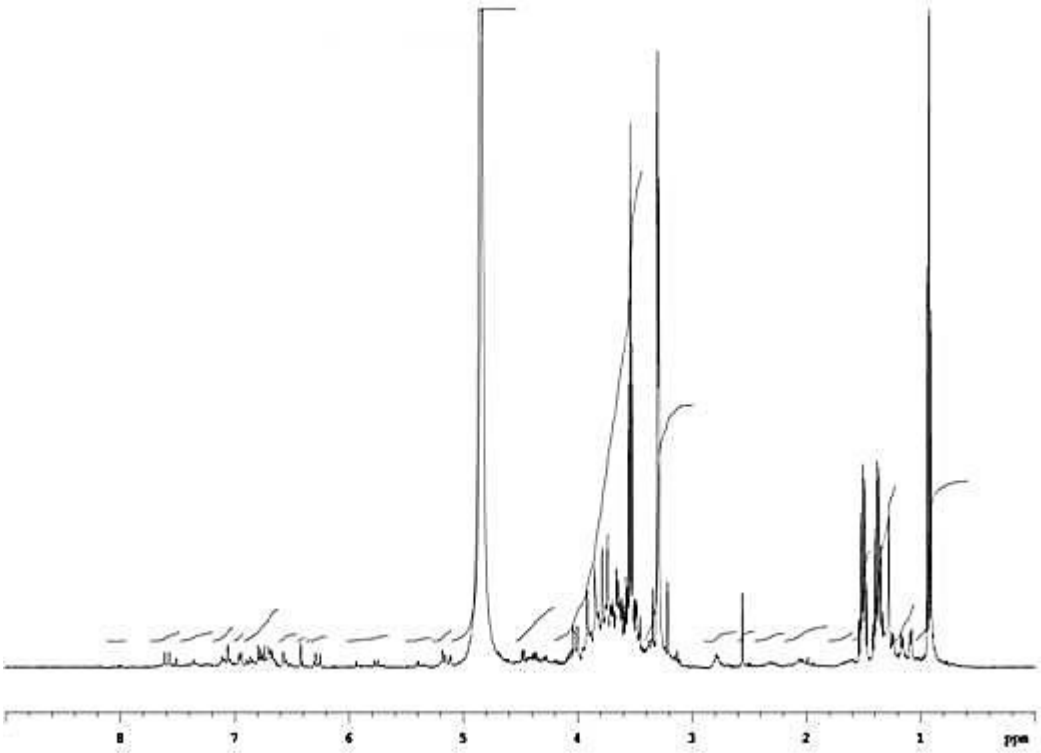
Appendix 8.1 ^{1}H NMR spectrum of *A. ebracteatus* in petroleum ether fraction (500 MHz, CD_3OD).



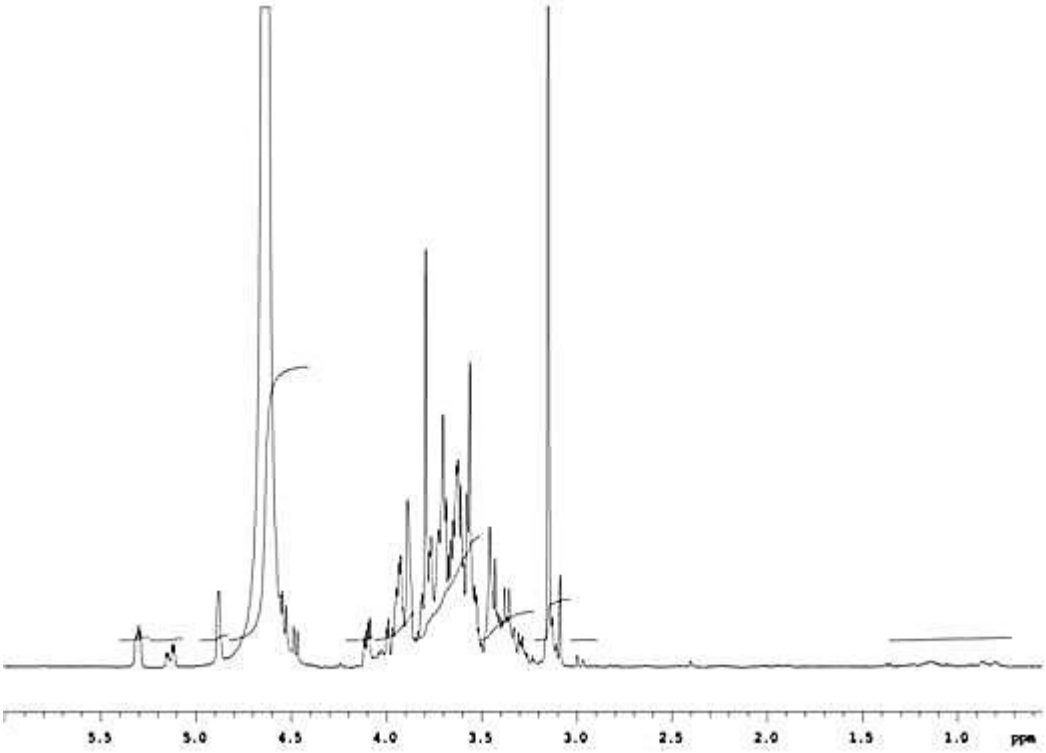
Appendix 8.2 ^{1}H NMR spectrum of *A. ebracteatus* in ethyl acetate fraction (500 MHz, CD_3OD).



Appendix 8.3 ^{1}H NMR spectrum of *A. ebracteatus* in butanol fraction (500 MHz, CD_3OD).

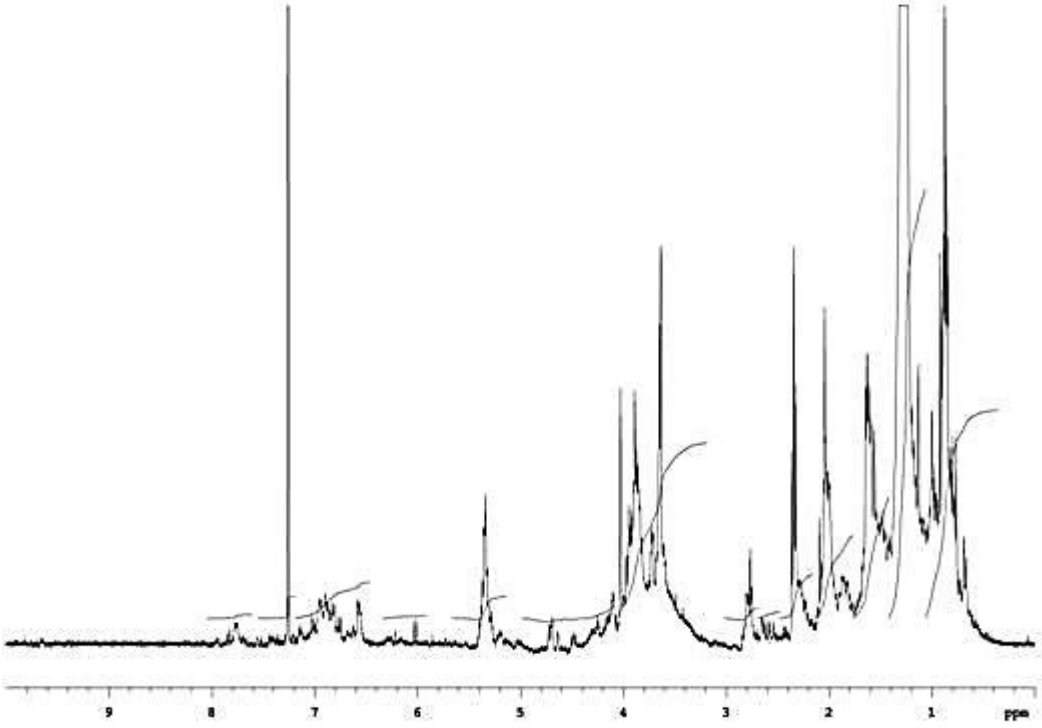


Appendix 8.4 ^{1}H NMR spectrum of *A. ebracteatus* in water fraction (500 MHz, CD_3OD).

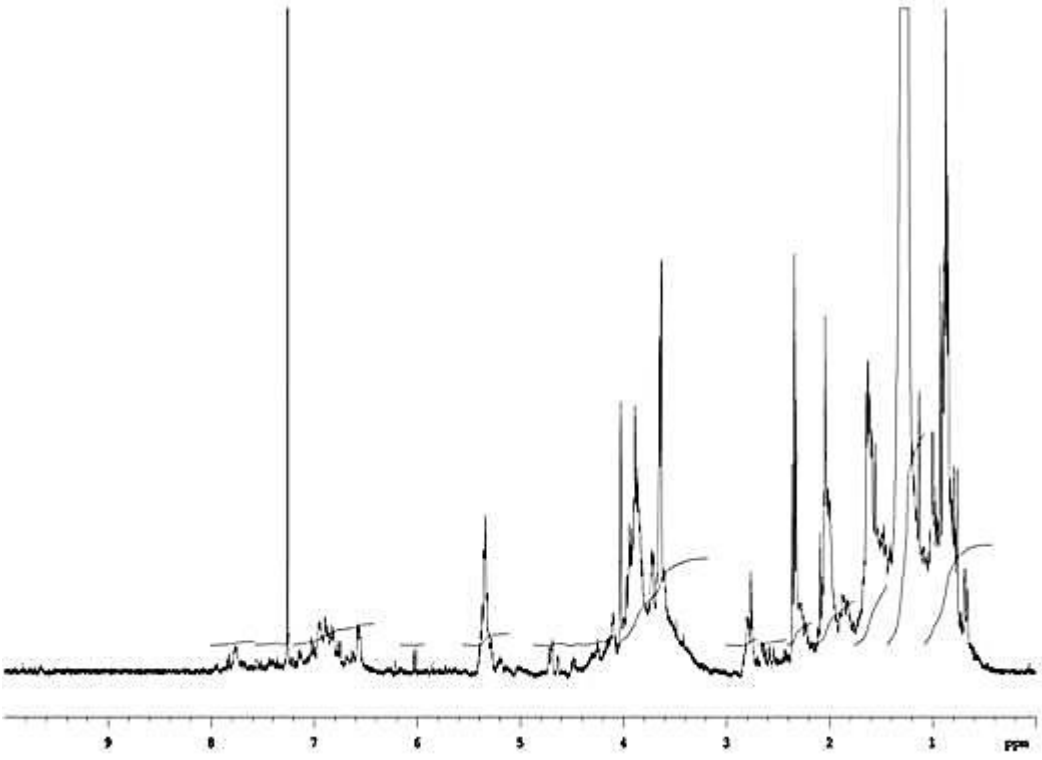


Appendix 9: NMR spectra of *C. petasites* in four fractions separated by liquid-liquid partition

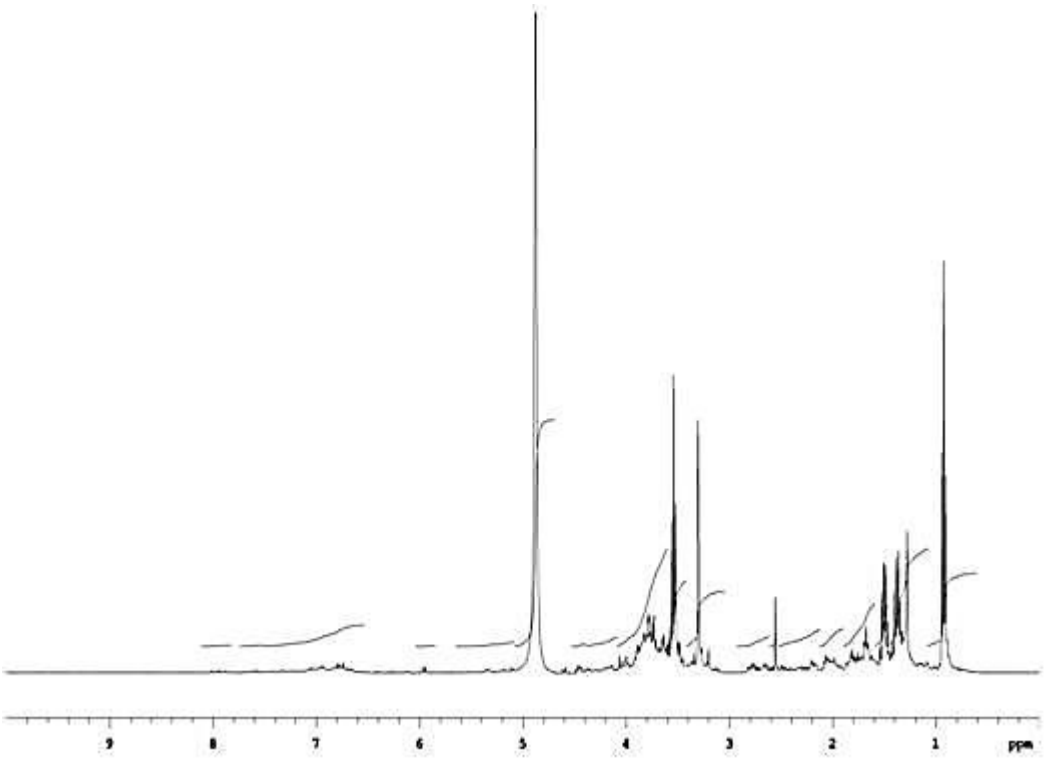
Appendix 9.1 ^{1}H NMR spectrum of *C. petasites* in petroleum ether fraction (500 MHz, CD_3OD).



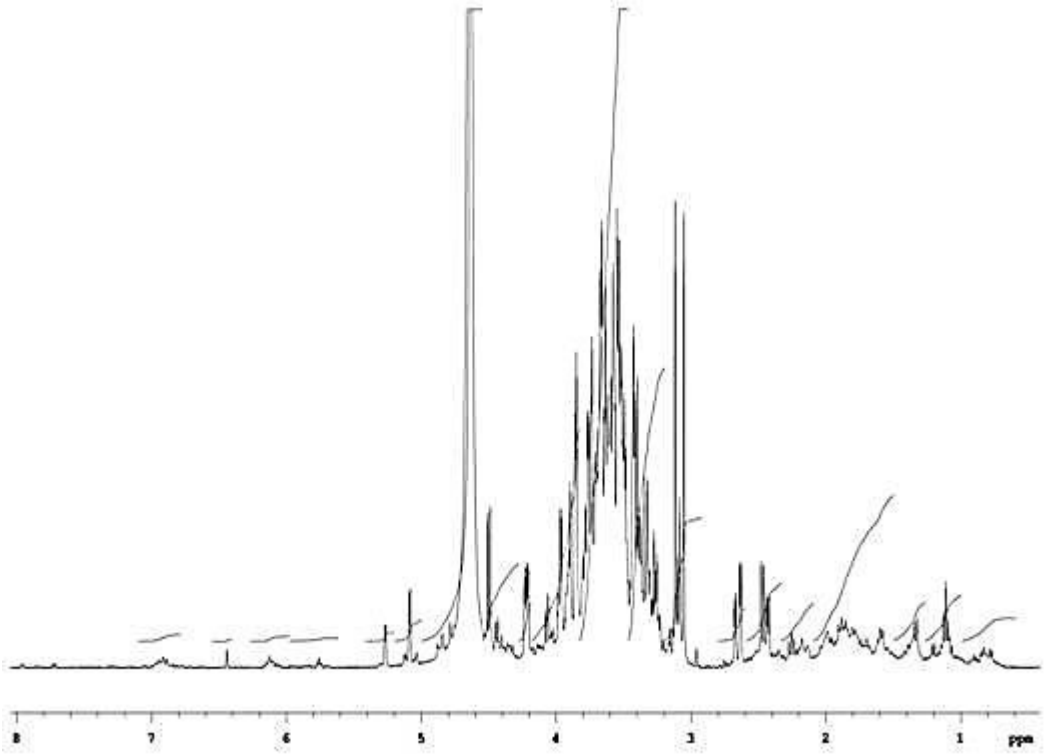
Appendix 9.2 ^{1}H NMR spectrum of *C. petasites* in ethyl acetate fraction (500 MHz, CD_3OD).



Appendix 9.3 ^{1}H NMR spectrum of *C. petasites* in butanol fraction (500 MHz, CD_3OD).

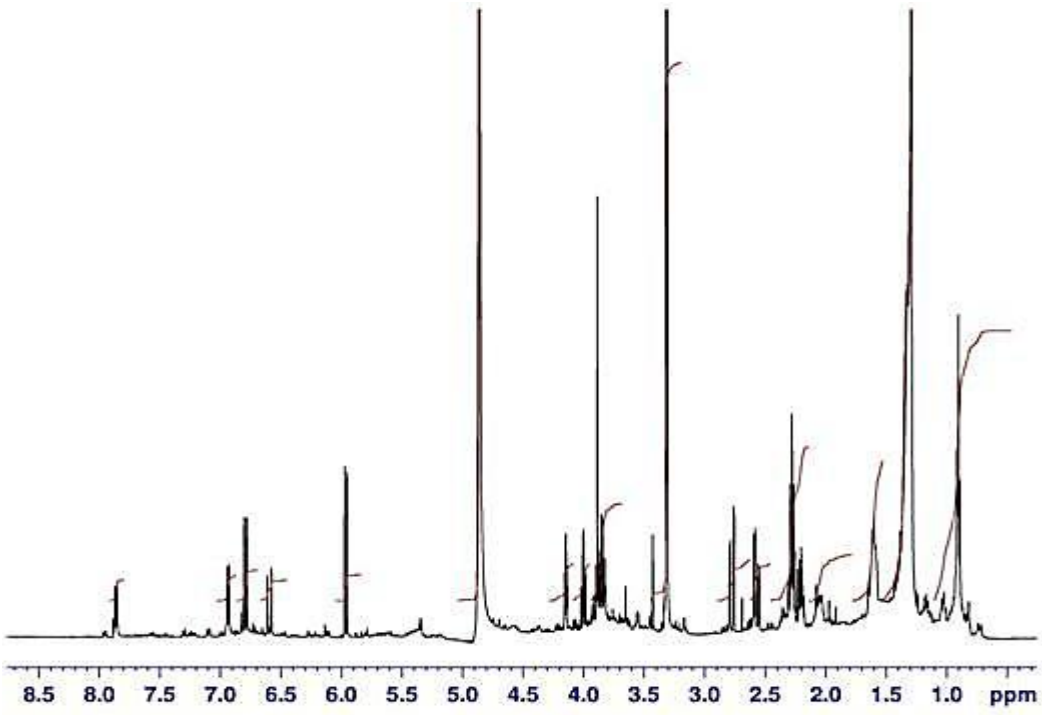


Appendix 9.4 ^{1}H NMR spectrum of *C. petasites* in water fraction (500 MHz, CD_3OD).

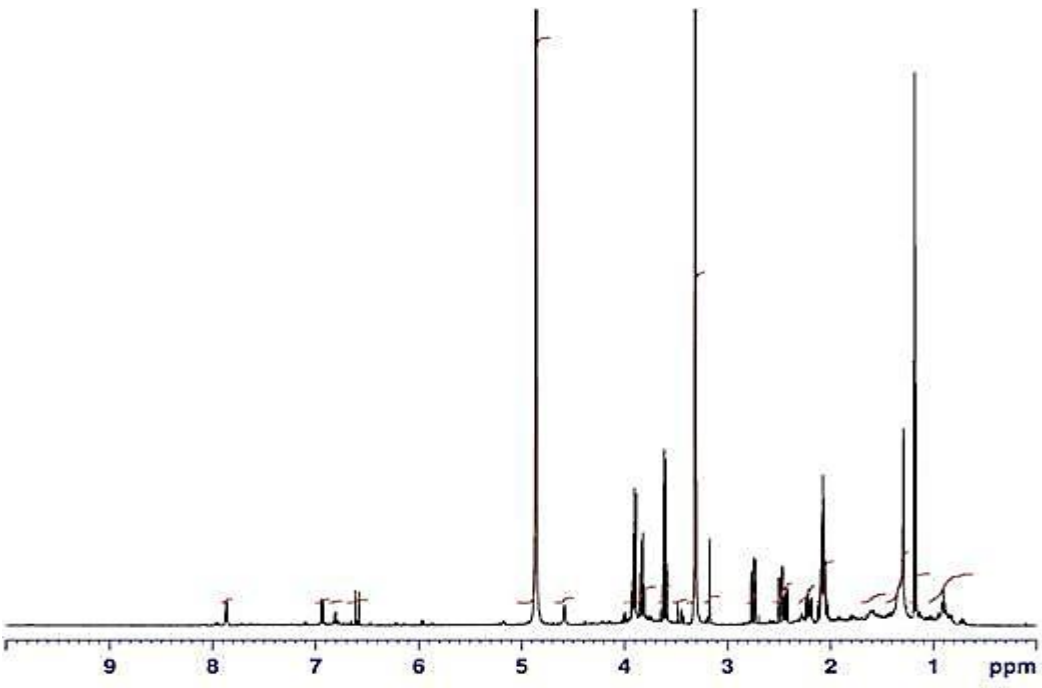


Appendix 10: NMR spectra of *C. petasites* in fractions separated by column chromatography

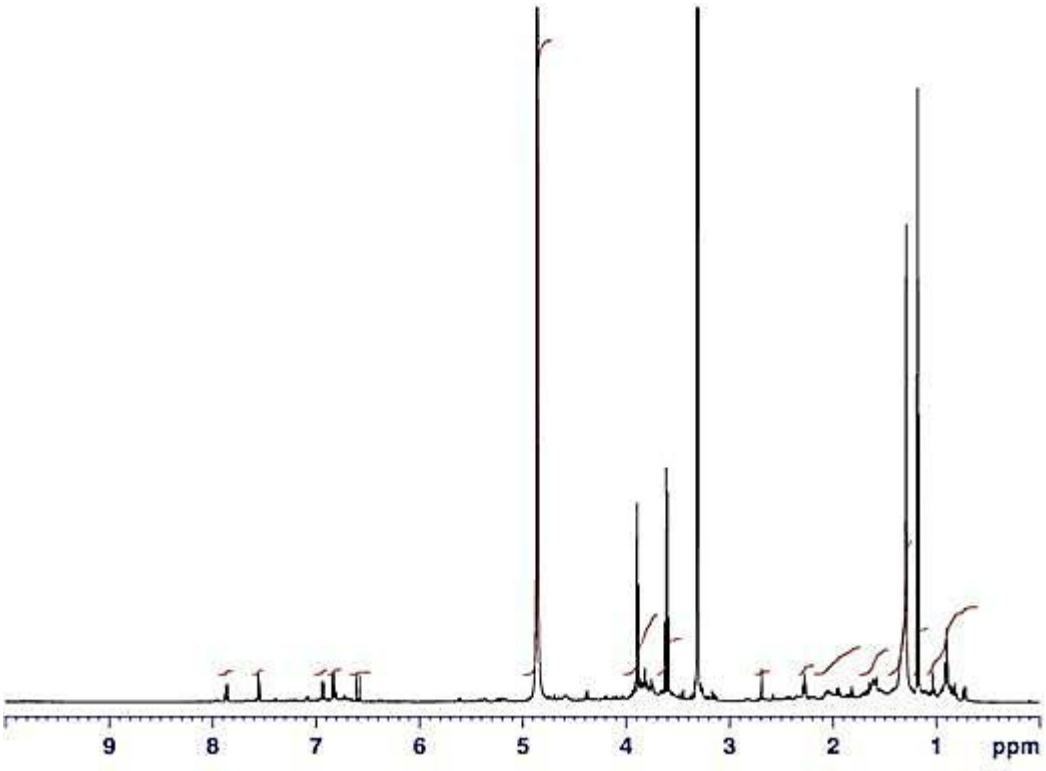
Appendix 10.1 ^{1}H NMR spectrum of *C. petasites* in a fraction separated by 100% ethyl acetate (500 MHz, CD_3OD).



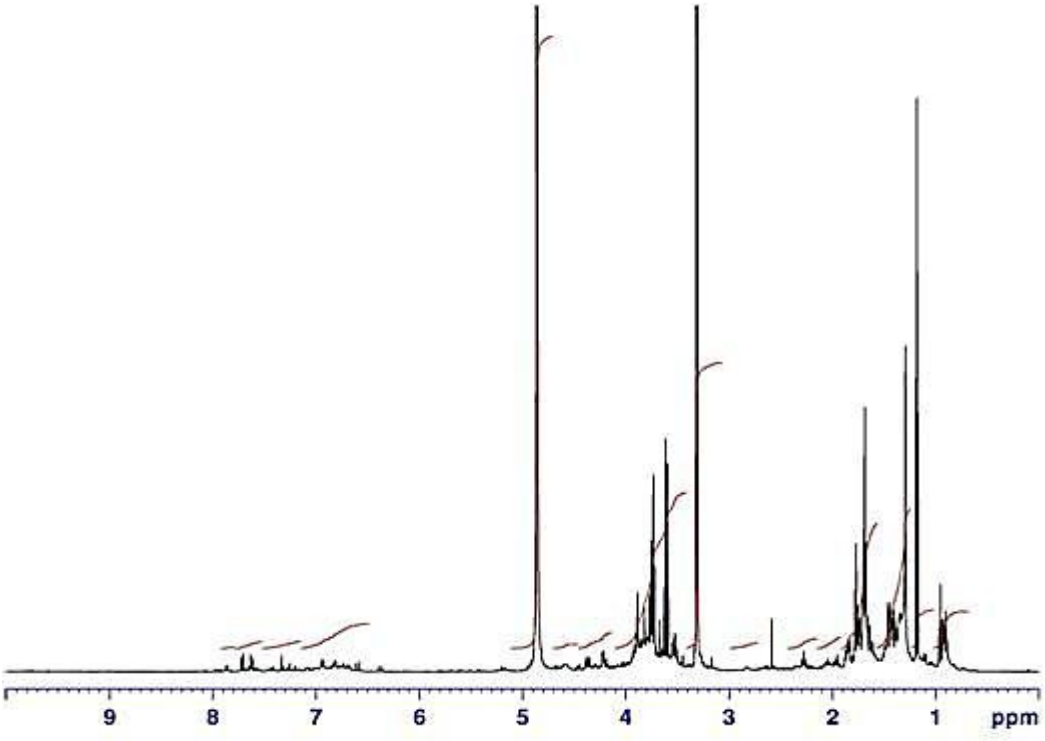
Appendix 10.2 ^{1}H NMR spectrum of *C. petasites* in a fraction separated by a mixture solution of 1% methanol in ethyl acetate (500 MHz, CD_3OD).



Appendix 10.3 ^{1}H NMR spectrum of *C. petasites* in a fraction separated by a mixture solution of 2% methanol in ethyl acetate (500 MHz, CD_3OD).



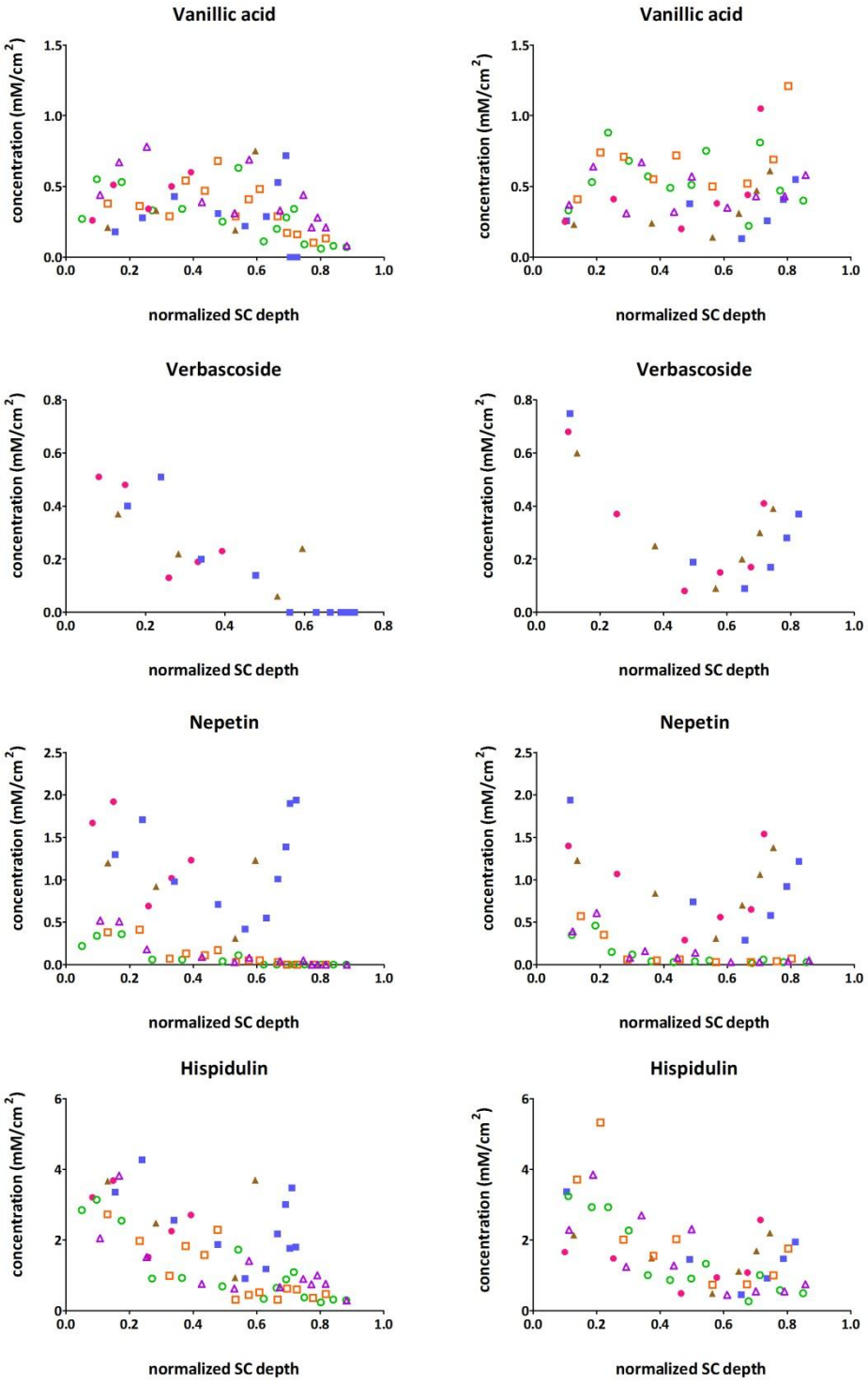
Appendix 10.4 ^{1}H NMR spectrum of *C. petasites* in a fraction separated by a mixture solution of 5% methanol in ethyl acetate (500 MHz, CD_3OD).



Appendix 11: *In vitro* SC concentration versus depth and SC distribution profiles

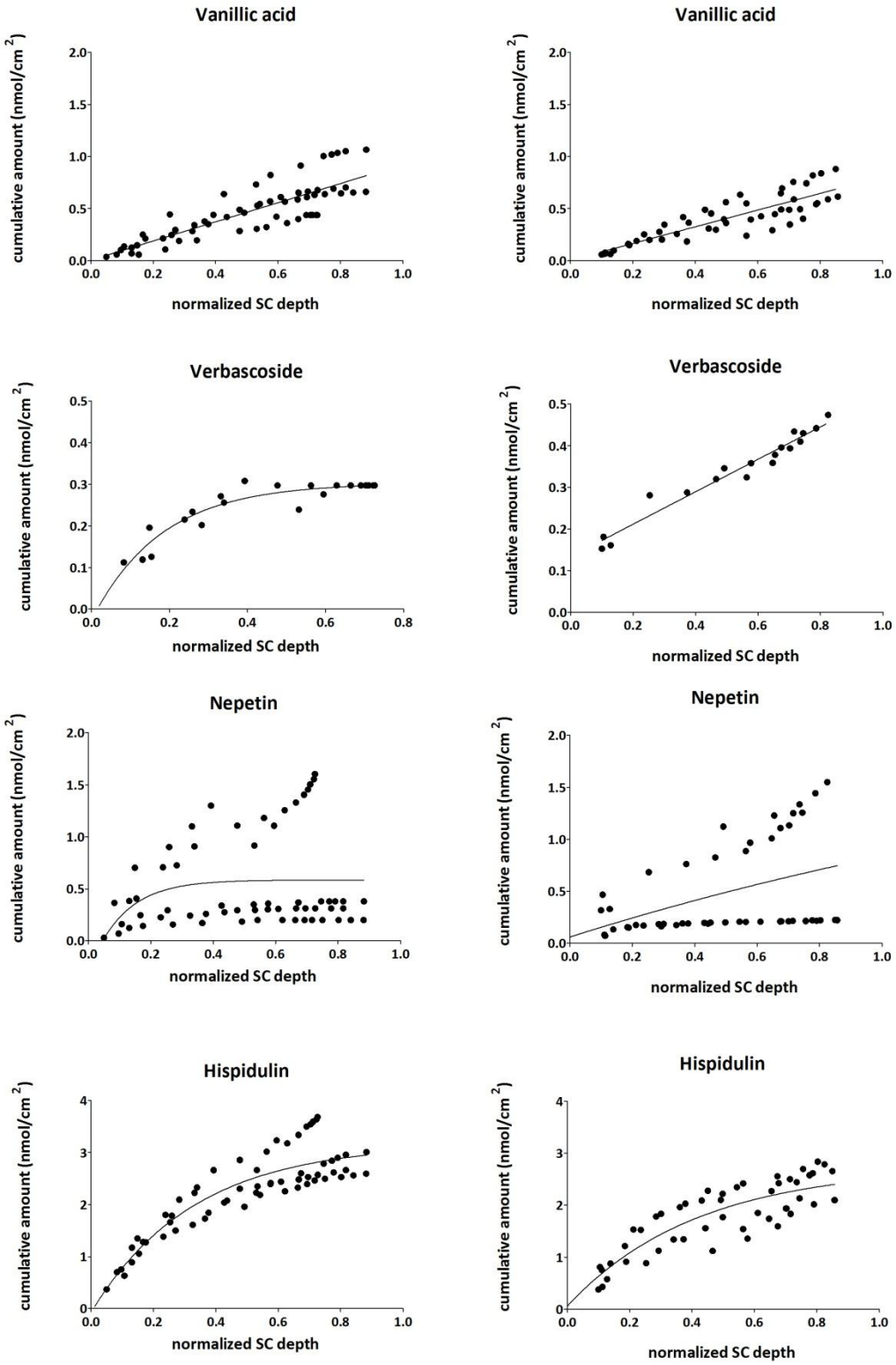
Appendix 11.1 *In vitro* SC concentration versus depth profiles of vanillic acid, verbascoside, nepetin and hispidulin (n = 6) after a 6-hour application of 10% w/w CP cream (panel A) and 10% w/w CP lotion (panel B). Each symbol represents data from a different cell.

A: 10% w/w CP cream **B: 10% w/w CP lotion**



Appendix 11.2 *In vitro* SC distribution profiles of vanillic acid, verbascoside, nepetin and hispidulin (n = 6) after a 6-hour application of 10% w/w CP cream (panel A) and 10% w/w CP lotion (panel B).

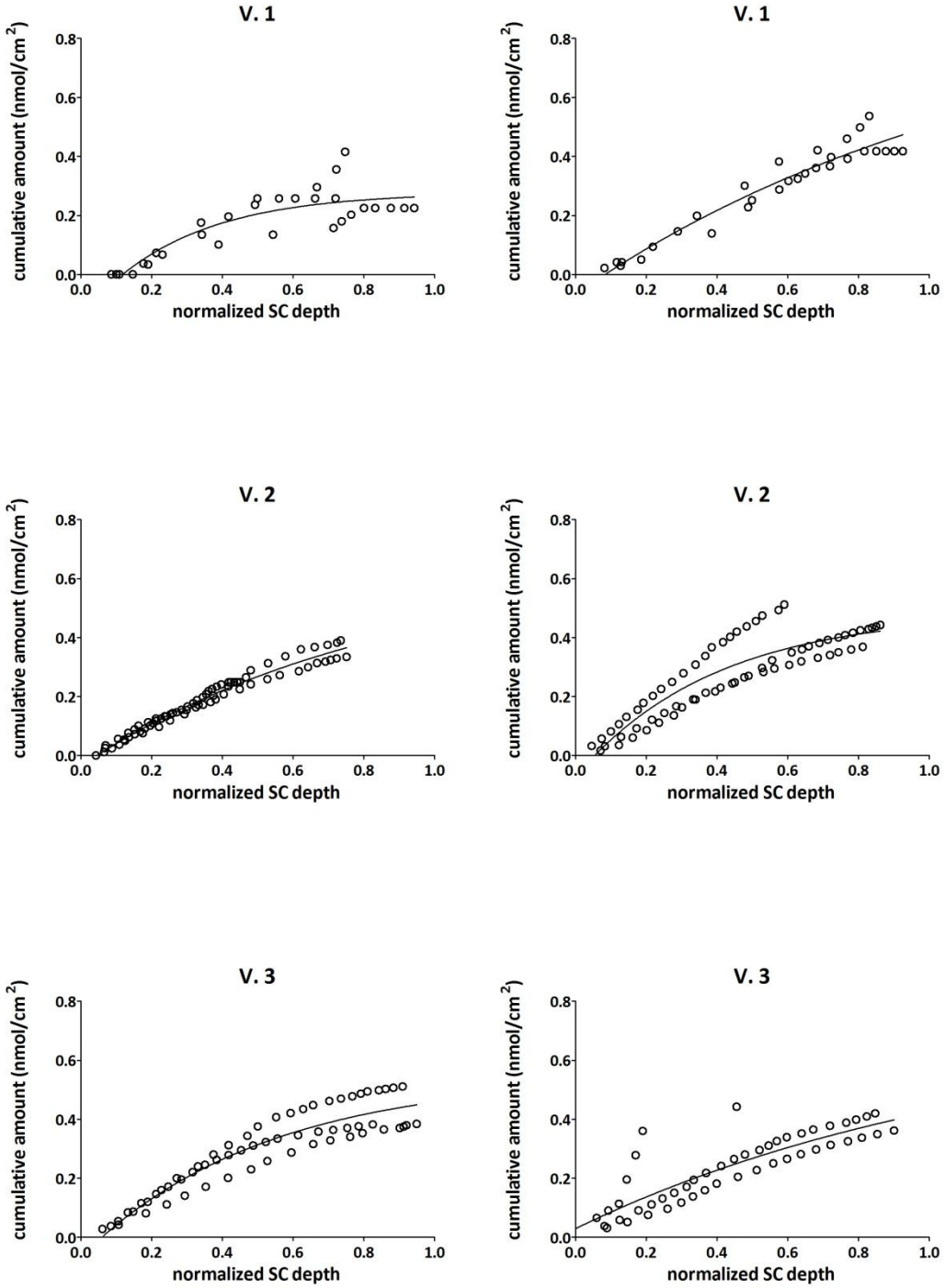
A: 10% w/w CP cream **B: 10% w/w CP lotion**



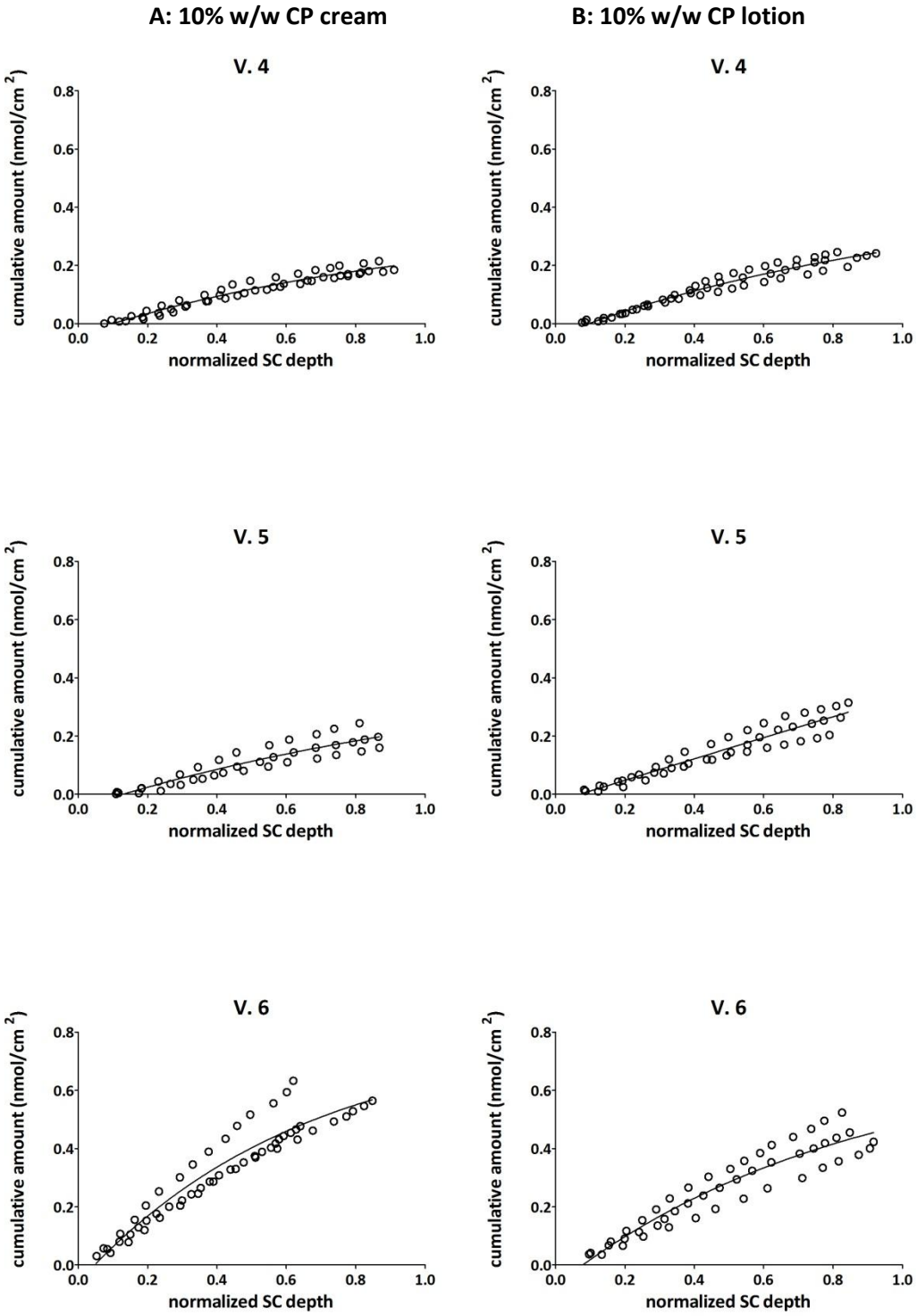
Appendix 12: *In vivo* SC distribution profiles

Appendix 12.1 Cumulative amount of vanillic acid against normalised SC depth in V. 1-3 after a 6-hour application of 10% w/w CP cream (panel A) and 10% w/w CP lotion (panel B):- continued overleaf.

A: 10% w/w CP cream **B: 10% w/w CP lotion**

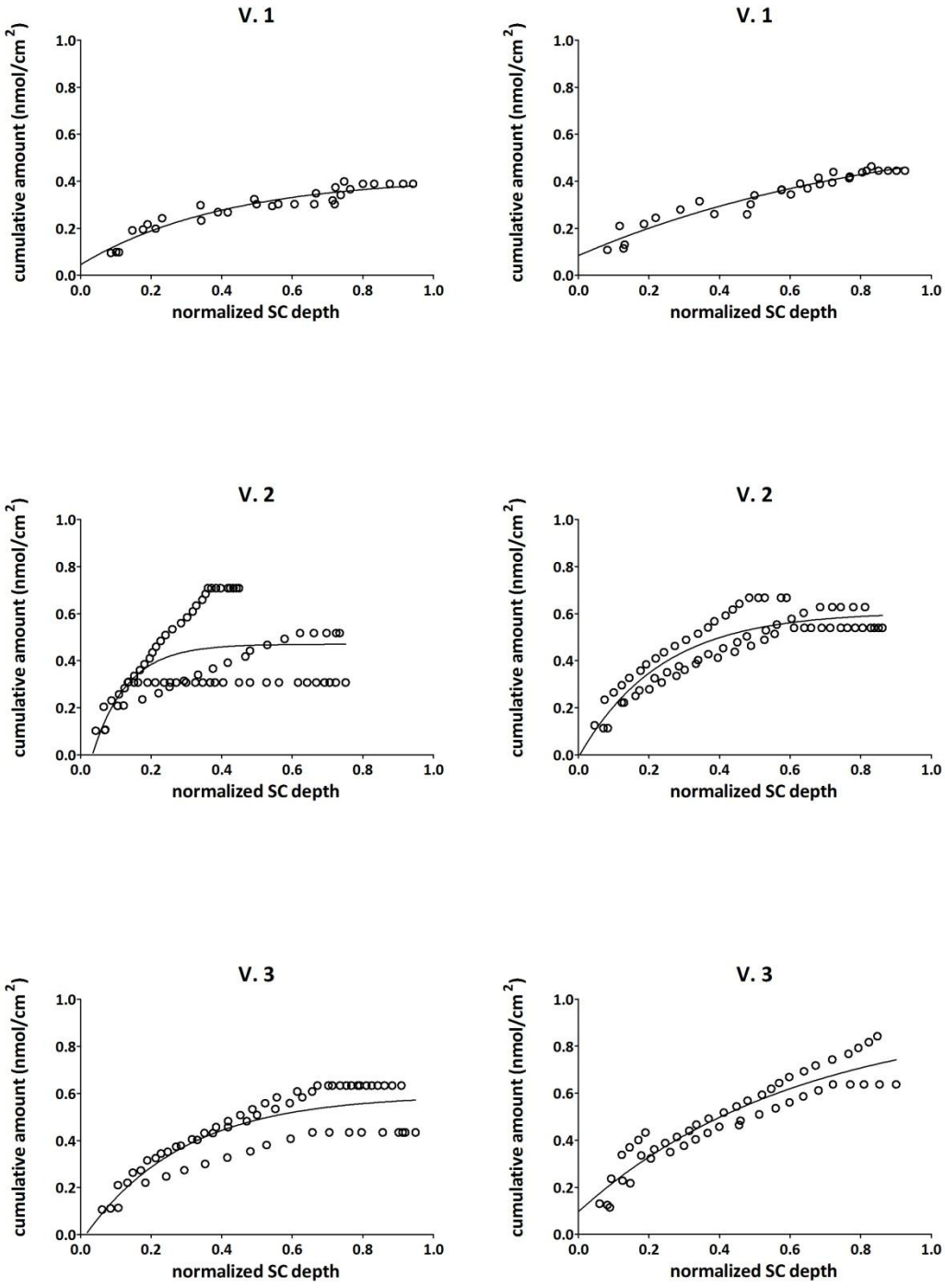


Appendix 12.1 cont. Cumulative amount of vanillic acid against normalised SC depth in V. 4-6 after a 6-hour application of 10% w/w CP cream (panel A) and 10% w/w CP lotion (panel B).



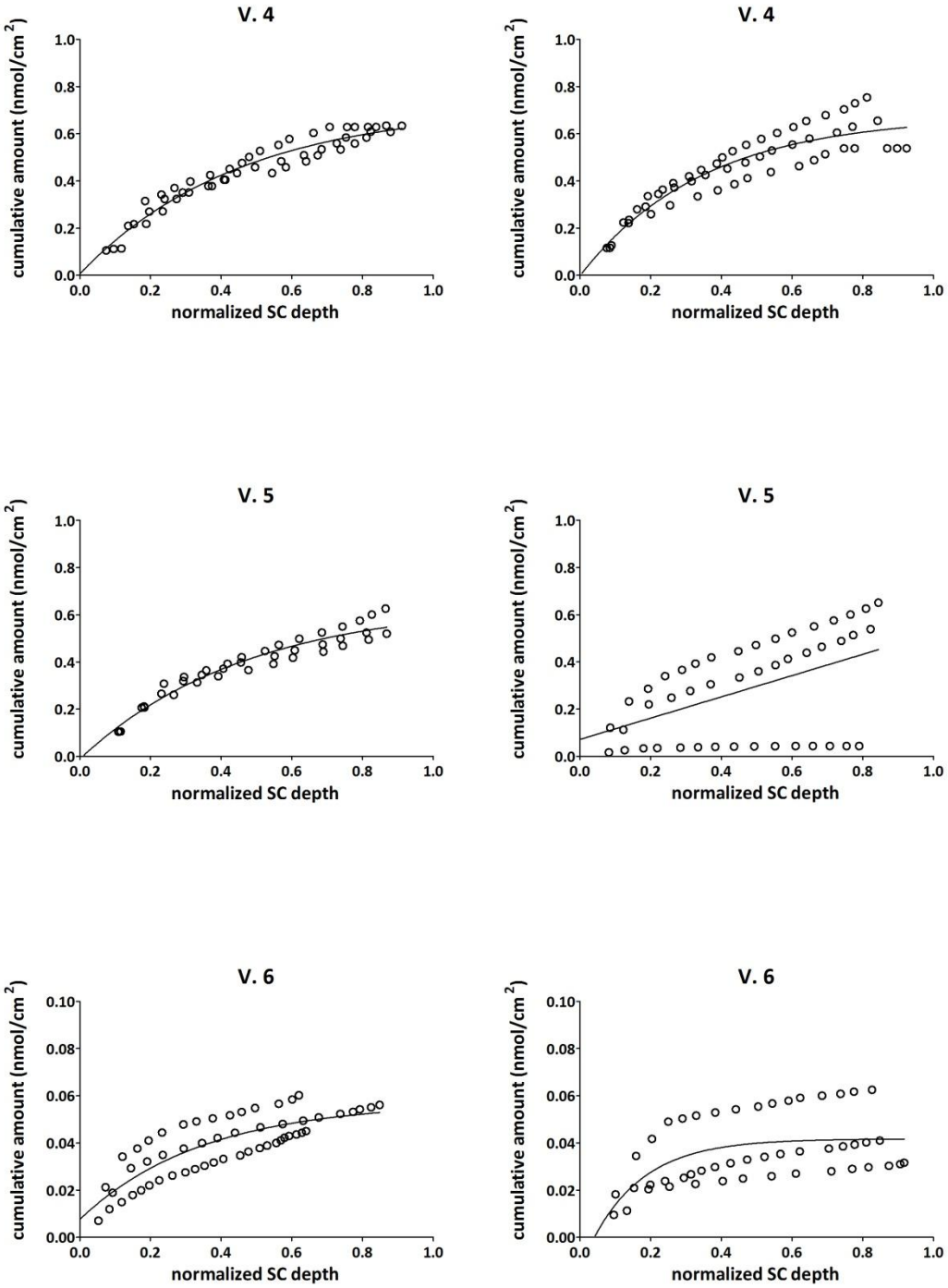
Appendix 12.2 Cumulative amount of nepetin against normalised SC depth in V. 1-3 after a 6-hour application of 10% w/w CP cream (panel A) and 10% w/w CP lotion (panel B):- continued overleaf.

A: 10% w/w CP cream **B: 10% w/w CP lotion**



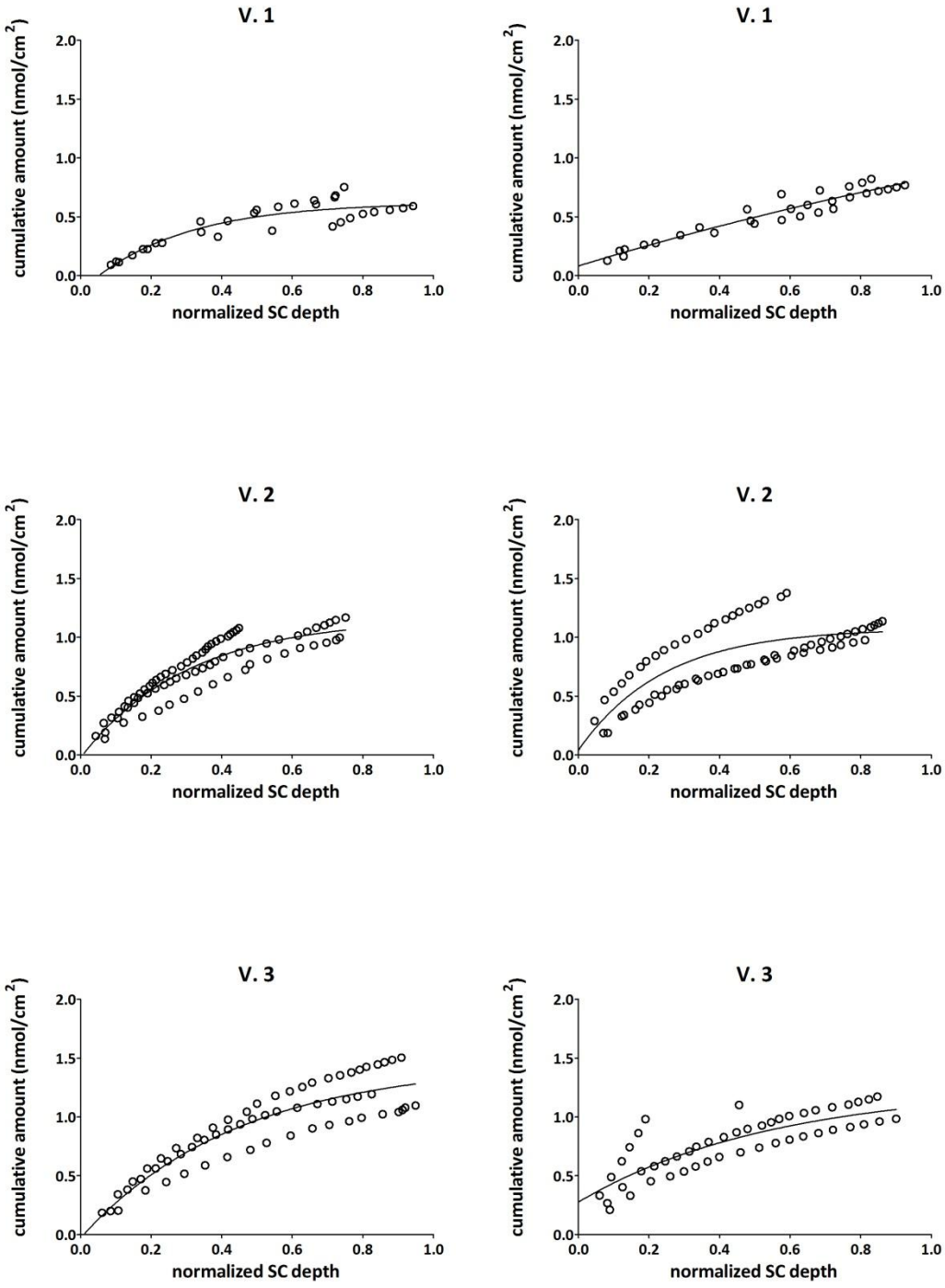
Appendix 12.2 cont. Cumulative amount of nepetin against normalised SC depth in V. 4-6 after a 6-hour application of 10% w/w CP cream (panel A) and 10% w/w CP lotion (panel B).

A: 10% w/w CP cream **B: 10% w/w CP lotion**



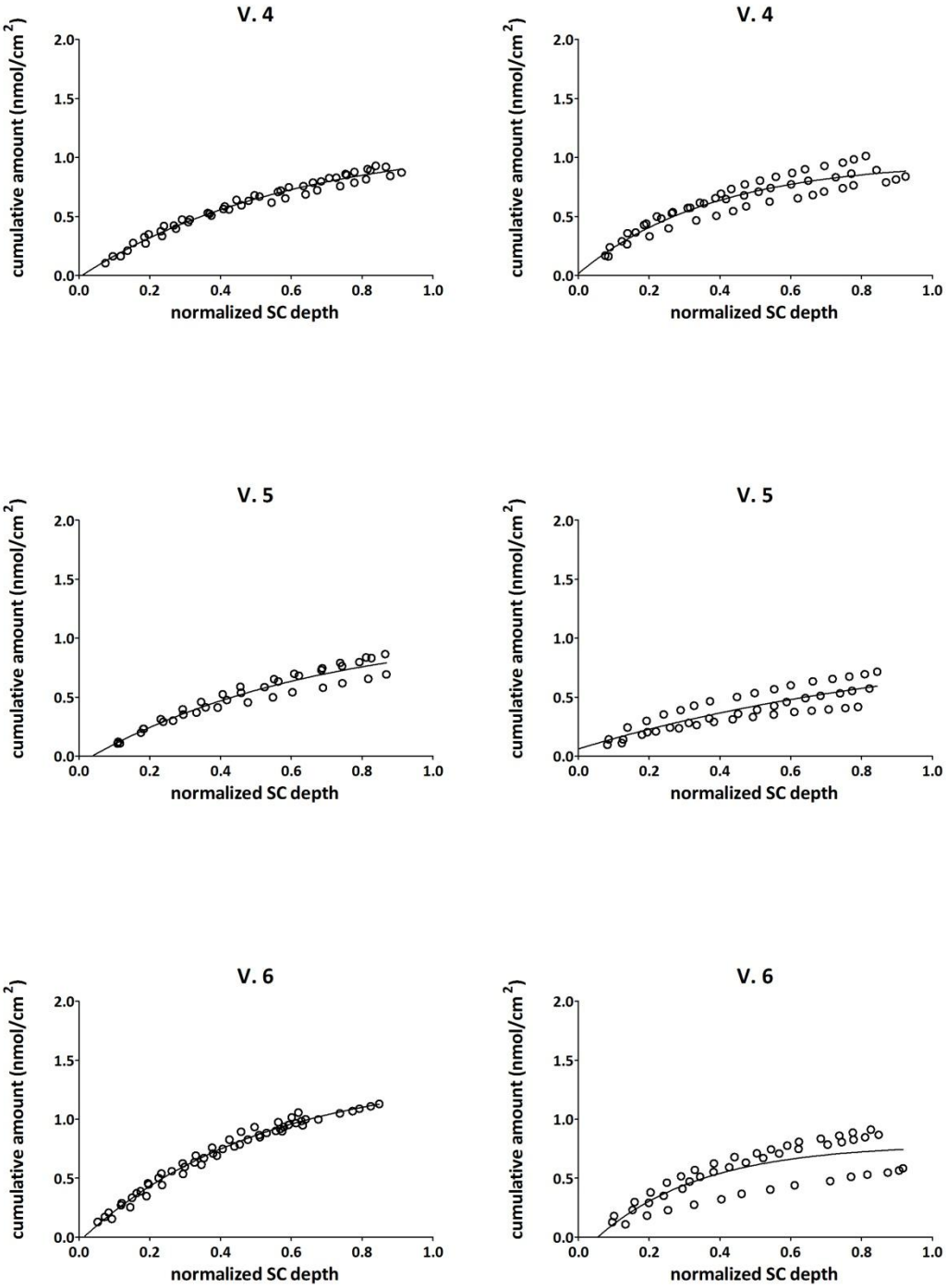
Appendix 12.3 Cumulative amount of hispidulin against normalised SC depth in V. 1-3 after a 6-hour application of 10% w/w CP cream (panel A) and 10% w/w CP lotion (panel B):- continued overleaf.

A: 10% w/w CP cream **B: 10% w/w CP lotion**



Appendix 12.3 cont. Cumulative amount of hispidulin against normalised SC depth in V. 4-6 after a 6-hour application of 10% w/w CP cream (panel A) and 10% w/w CP lotion (panel B).

A: 10% w/w CP cream **B: 10% w/w CP lotion**



Appendix 13: Contents of phenolic makers of *C. petasites* in ethanolic extracts after 4-year storage

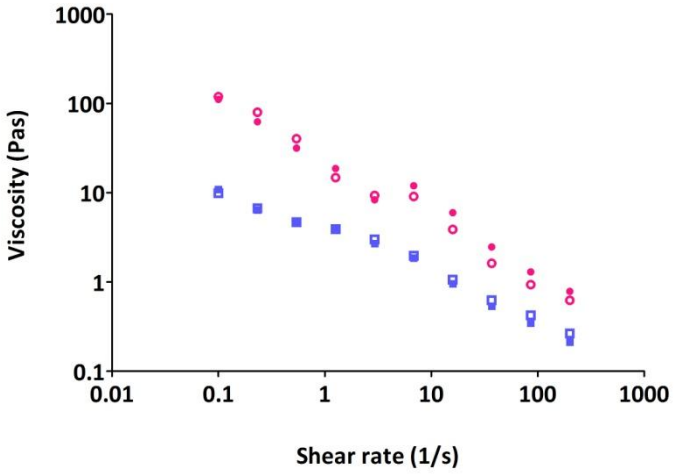
*the dried ethanolic extracts were kept in a desiccator at room temperature.

		Amount (µmol/g) in dried extract of *C. petasites*			
	Batch no.	**Vanillic acid**	**Verbascoside**	**Nepetin**	**Hispidulin**
Fresh extracts	1	3.4	4.8	17.9	35.7
	2	2.5	3.9	12.8	29.8
	3	3.7	5.3	17.4	49.3
	4	2.0	2.9	11.5	35.0
	5	2.0	2.5	12.8	42.5
	Average ± SD	*2.7 ± 0.8*	*3.9 ± 1.2*	*14.5 ± 2.9*	*38.5 ± 7.6*
4-year stored extracts	1	4.0	4.3	3.2	27.3
	2	4.0	4.2	3.3	32.1
	3	4.2	4.0	2.9	29.0
	4	3.7	3.5	2.3	29.8
	5	4.1	3.8	2.9	28.7
	Average ± SD	*4.0 ± 0.3*	*4.0 ± 0.4*	*2.9 ± 0.4*	*29.4 ± 2.1*

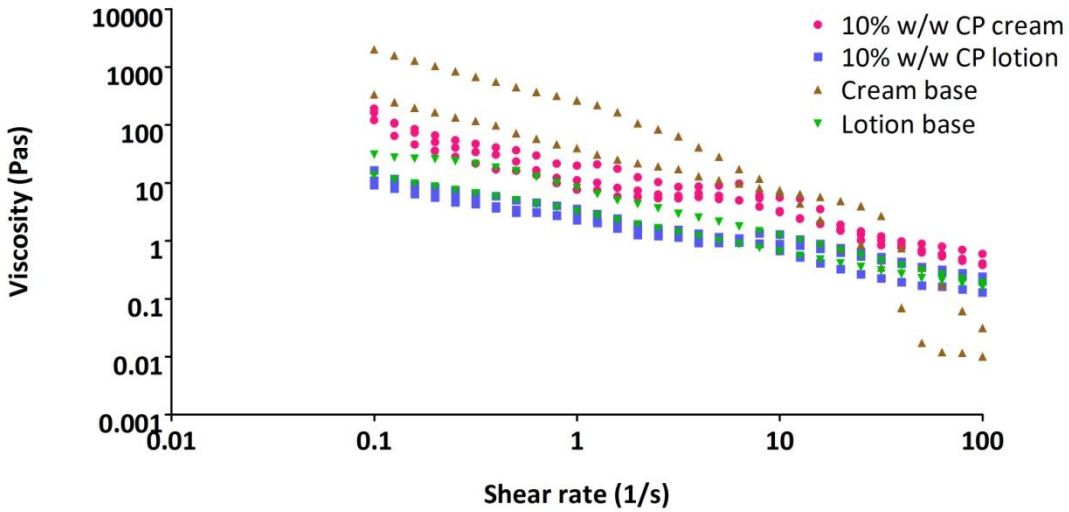
Appendix 14: Viscosity profiles of lotion and cream containing 10% w/w C. petasites

Instrument and condition: cone angle 4° and diameter 40 mm with 0.15 mm gap at 27°C, Bohlin rheometer, Malvern Instruments Ltd, Worcestershire, UK.

Appendix 14.1 Viscosity profiles of 10% w/w CP cream and lotion before and after reversible separation. Pink and blue colours represent 10% w/w CP cream and lotion, respectively. Bold and blank circles respectively represent before and after reversible separation of the formulations.



Appendix 14.2 Viscosity profiles of 10% w/w CP cream, lotion and their bases from different batches. Each data set represents viscosity from a different batch of formulation. Non-Newtonian and shear thinning behaviour are shown.



Appendix 15: Ethics

Appendix 15.1 Ethics application form.

ANNEX ONE

School for Health

School Research Ethics Approval Panel

Checklist for all researchers

The School for Health requires all members of staff and students who are planning research projects to consider the ethical implications of the work which they undertake. This is important in all research projects, but is ***essential*** in those projects which involve human participants.

The School has agreed on an ethical review process which has a fast track for those projects which either do not have ethical implications and thus do not require full scrutiny, or where scrutiny will be given by another body (in particular an NHS Research Ethics Committee [REC]) or other UK University. Projects which fall outside of these categories will need to make a full submission to the School Research Ethics Approval Panel.

Name	**Premrutai Thitilertdecha, Dr. Michael G Rowan, Prof. Richard H Guy**
Project title	***In vivo* assessment of a topical application of *Clerodendrum petasites* S. Moore**

Determining the nature of your research and the route for ethical approval you need to follow

(please tick the route you will follow for your ethical approval):

My research project has been successful in receiving external funding by the ESRC ☐

(full consideration is required by the SSREC. Further details can be obtained from:

http://www.bath.ac.uk/internal/research/ssrec/. For audit purposes a copy of the

SSREC application & decision letter as well as this form and EIRA1 will need to

be returned to the School for Health Administrator) (Annex 3)

My proposal is currently at the stage of application for funding (tick box) ☐

Please complete annex 1 & 2 for SREAP audit purposes. Further approval may be

required once funding is approved (please refer to relevant statement below).

My research project does not involve the use of human subjects ☐

(full consideration is not required, complete the checklist and the implications form for audit purposes and return to the School Administrator; Principal investigator, second reader and researcher to sign and return to the School Administrator (Annex 2 or 3))

My research meets the requirements for submission to an NHS REC ☐

(e.g. Involves human subjects, requires access to NHS patients or will be conducted on NHS premises) (full consideration is required by the appropriate NHS REC; complete the checklist and ethical implications form for audit purposes and return to the School Administrator (Annex 2 or 3) together with the evidence of NRES approval)

My research has received approval from another UK University ethics committee. ☐

Complete the checklist and submit with evidence of the institutions approval, together with Annex 2 or 3

My research involves human subjects and does not take place in an NHS context ☑

(full consideration is required by SREAP (Annex 2 or 3 and Annex 4))

My research involves human subjects and takes place outside of the UK, and for which particular consideration needs to be given ☐

(full consideration is required by SREAP - Annex 2 or 3 and Annex 4))

My research involves working with children and/or vulnerable adults ☐

(a CRB check may also be required in addition to the above)

My research involves the collection and storage (for more than 48 hours) ☑

of human tissue. (Full consideration from an NRES approved committee is required in addition to the above)

ANNEX TWO

School for Health

EHICAL IMPLICATIONS OF POSTGRADUATE RESEARCH PROJECT

This template should accompany the postgraduate research student application for candidature form submitted to the Board of Studies. *(Additional departmental information may be incorporated as appropriate).*

Please note that this procedure is intended to help student and supervisor consider ethical implications of the proposed research project, and as such is a 'light-touch' approach. Supervisors are responsible for deciding whether a more extensive ethical review is necessary (by submission to the School Research Ethics Approval Panel or an external ethical approval body, such as the NHS REC).

Brief Title of Project	***In vivo* assessment of a topical application of *Clerodendrum petasites* S. Moore**
Student	**Premrutai Thitilertdecha**
Supervisor (s)	**Prof. Richard H Guy, Dr. Michael G Rowan**

Are there ethical implications concerned with the following general issues?

	Comments from Supervisor
Source of the funding	Faculty of Medicine Siriraj Hospital, Mahidol University, Thailand.
What steps will or have been taken to ensure competency of the student?	Student has been trained by competent postdoctoral and technical staff (in the PI's laboratory) who have been working on LREC-approved projects using similar experimental techniques.
Are there any Data storage issues? (including confidentiality, availability, length of storage, etc)	Participants will be given a code name. Only the researcher (Premrutai Thitilertdecha) and supervisors will have access to the identities of the participants. The information will not be stored on the C drive of any individual computer. Photographs may be taken of the volunteers' forearms during their participation. The photos will be restricted to the areas of treatment on the forearm and will not allow identification of the participant; informed verbal consent will be obtained prior to any photos being taken. Agreeing to have the photos taken is not a condition of

	participation. Premrutai Thitilertdecha will maintain the security of all data during the study. Once the study is finished, the records will be securely stored at the University of Bath under control of the PI. General public and the scientific community will have access to the data in the form of presentations & posters at meetings, and via scientific articles which will be published.
Dissemination of results: 1) Are any ethical issues likely to arise? 2) Are there appropriate plans for the dissemination?	Anonymised data only will be transmitted via email and computer networks. These data will not include any person-identifiable information. Presentations, articles, and any other form of communication of the data obtained will avoid any detail that could result in the identification of the participants.
Effect on/damage to the environment	N/A
In which aspects of the research process have you actively involved, or will you involve patients, service users, or members of the public?	*Please tick all that apply* ☐ Design of the research ☐ Management of the research ☒ Undertaking the research ☐ Analysis of results ☐ Dissemination of findings ☐ None of the above
Give details of patient, service users or public involvement, or if none please justify the absence of involvement.	The research is not sufficiently advanced to warrant patient participation at this time.

Demonstration of Ethical Considerations

To be completed by the student and supervisor. Please provide a paragraph describing the ethical issues which will need to be managed during the course of the activity. See overleaf for possible areas for consideration.

1) Confidentiality. This has already been addressed.

2) Healthy volunteers. Volunteers will be involved in the study and will gain no benefit from their participation. They will receive a nominal payment to compensate for any inconvenience caused. Payment has been proposed at a level which we hope will produce interest but low enough to avoid coercion.

3) Exposure to creams/lotions: During the course of the experiment, participants will be asked to inform us if they experience any unexpected adverse effects. If ongoing monitoring indicates persistent severe reactions to the plant extract (*Clerodendrum petasites* S. Moore) or any excipients in the formulations, the exposure will be stopped and the formulations applied will be removed.

4) Invasive procedures: Volunteers will undergo exposure to creams/lotions, which may cause some local irritation if the volunteers are partially sensitive to excipients in the formula. However, there have been no reports of toxicity or adverse drug reactions involving *C. petasites* and the excipients are commonly used in cosmetics and topical products. All experimental protocols and risks are detailed in the patient information leaflets.

5) Invasive procedures: Post-application and removal of the formulations, the surface skin layers at the treatment site will be progressively removed by the adherence and swift removal of sticky tape. This procedure may cause very slight discomfort. To mitigate against this, measurements of skin barrier function are made between each tape-strip to ensure that no more than about 60% of the skin barrier is removed before tape-stripping is terminated.

Issues for additional consideration: (*This list is indicative and is not necessarily exclusive). Please tick which categories apply to your research.*

	Yes	**No**
1. Does the study involve participants who are particularly vulnerable or unable to give informed consent? (eg children, people with learning disabilities)		√
2. Will the study require the co-operation of a gatekeeper for initial access to the groups or individuals to be recruited? (eg students at school, members of self-help group, residents of a nursing home)		√
3. Will it be necessary for participants to take part in the study without their knowledge and consent at the time? (eg covert observation of people in non-public places)		√
4. Will the study involve discussion of sensitive topics? (eg sexual activity, drug use)		√
5. Are drugs, placebos or other substances (eg food substances, vitamins) to be administered to the study participants or will the study involve invasive, intrusive or potentially harmful procedures of any kind?	√	
6. Will blood or tissue samples be obtained from participants?	√	
7. Is pain or more than mild discomfort likely to result from the study?		√
8. Could the study induce psychological stress or anxiety or cause harm or negative consequences beyond the risks encountered in normal life?		√
9. Will the study involve prolonged or repetitive testing?	√	
10. Will financial inducements (or other reasonable expenses and compensation for time) be offered to participants?	√	
11. Will the study involve recruitment of patients or staff through the NHS? (Note: If the answer to this question is 'yes' you will need to submit an application to appropriate NHS Research Ethics Committee.)		√
12. Will the study involve obtaining or processing personal data		√

	Yes	No
relating to living individuals, (eg involve recording interviews with subjects even if the findings will subsequently be made anonymous)? (Note: If the answer to this question is 'yes' you will need to ensure that the provisions of the Data Protection Act are complied with. In particular you will need to seek advice to ensure that the subjects provide sufficient consent and that the personal data will be properly stored, for an appropriate period of time). Information is available from the University Data Protection Website and dataprotection-queries@bath.ac.uk		
13. Will the study involve the use of animals?		√
14. Does the study raise any other ethical issues which you wish to be raised and reviewed by the School Research Ethics Approval Panel? If yes, what are they, please expand here:		√

I confirm that the statements above describe the ethical issues which will need to be managed during the course of this research activity.

Researcher/Student	**Signature:** **Date:**

I confirm that the details given in this form accurately describe the ethical issues which will need to be managed during the course of this research activity. I am satisfied with the scientific design and integrity of this project.

Principal Investigator/ Supervisor/ Project Supervisor	**Signature:** **Date:**
Second reader **(PhD/DHealth/MPhil/MD only)** *(This will normally be a person external to the project team)*	**Signature:** **Date:**

Please submit this form to the School Administrator.

PROCESS

1. Completion of template *Ethical Implications of Research Activity* by Principal Investigator (PI) for all research proposals (to accompany RS1). PI passes the template to a Second Reader and then on to the Head of Department.

2. Completed signed template to be passed to Departmental Research Ethics Officer who will review issues for action and inclusion in Departmental Annual Report.

3. If any significant issues arise during the project they should be investigated by the Department and, if appropriate, forwarded to the University Ethics Committee for guidance. At the end of the project, to reflect good departmental practice, the PI will report to the Departmental Research Ethics Officer if ethical issues changed during the course of the project.

4. The Head of Department will prepare an Annual Report for submission via Departmental/Faculty Research Committees for review and to the University Ethics Committee for monitoring purposes (standard template to be provided). Ethics Committee will report to Senate via minutes.

You must submit your project to an NHS Local Research Ethics Committee (LREC) if it involves any of the following:

- Patients and users of the NHS. This includes all potential research participants recruited by virtue of the patient or user's past or present treatment by, or use of, the NHS. It includes NHS patients treated under contracts with private sector institutions.
- Individuals identified as potential research participants because of their status as relatives or carers of patients and users of the NHS, as defined above.
- Access to data, organs or other bodily material of past and present NHS patients.
- Fetal material and IVF involving NHS patients.
- The recently dead in NHS premises.
- The use of, or potential access to, NHS premises or facilities.
- NHS staff - recruited as research participants by virtue of their professional role.

Bath Research Ethics Contact: Vanessa Bishop / Anna Jenkins.

Room 11, John Apley Building, Royal United Hospital, Combe Park, Bath, BA1 3NE.

Tel: 01225 825725. Fax: 01225 825725. E-mail: vanessa.bishop@ruh-bath.swest.nhs.uk

However if the research is at more than one site (geographically) then approval has to be through a REC allocated through the central system.

See http://www.corec.org.uk/applicants/apply/cas.htm.

THIS NOTES PAGE IS FOR INFORMATION ONLY AND MAY BE DETACHED FROM THE FORM IF REQUIRED

ANNEX FOUR –Application form for full submission for research ethics approval

School for Health

School Research Ethics Approval Panel

Full Application for Consideration for Research Ethics Approval

Title of study	***In vivo* assessment of a topical application of *Clerodendrum petasites* S. Moore.**
Chief investigator **(for research student projects, put research supervisors name here)** **(for undergraduate projects, put project supervisors name here)**	Name: Prof. Richard H Guy e-mail: R.H.Guy@bath.ac.uk Telephone: 01225384901 Name: Dr. Michael G Rowan e-mail: M.G.Rowan@bath.ac.uk Telephone: 01225386789
Other investigators **(for research student projects, put students name here)** **(for undergraduate projects, put student(s) name here)**	Name: Premrutai Thitilertdecha e-mail: pt245@bath.ac.uk Telephone:01225383900
Source of funding for the study	University of Bath
Proposed dates of study	1st January 2012 to 1st January 2013
Research question	The project primarily aims to assess whether formulating *C. petasites* extracts into creams/lotions will enhance the penetration of naturally-occurring components (mainly polyphenolic compounds which are well-known for antioxidant activity) through the stratum corneum (SC) in term of penetration rate and amount. The obtained results may support Thai traditional use of the plant and improve the quality and efficacy of a topical herbal product.
Background (less than 100 words)	*C. petasites* is commonly used in Thai traditional medicine for skin treatment (e.g., rash). However, only hispidulin and

	arbutin have been previously reported as chemical constituents and no studies on any optimised topical delivery system have been reported. In terms of dosage forms, only crushed leaves and powders are currently accessible and they are formulated as rather idiosyncratic poultices and decoctions with poor reliability in quality, safety and efficacy.
Methods (less than 300 words)	The experiment will be performed for 2 days using both right and left forearms of volunteers. On each arm, there will be 4 treated skin sites, three with the *C. petasites* (10% w/w) cream/lotion formulation, and one with a control, blank vehicle containing no actives. Approximately 0.2g of the product will be applied on each site (6 cm^2 in area) for 6 hours. During the exposure, the treatment site will be occluded. After that, stratum corneum (SC, the outermost layer of the skin) will be removed by tape strips (Permacel J-LAR®) and the removal of SC monitored by transepidermal water loss (TEWL, the flux of water loss through the skin). The tape stripping process will stop either when TEWL reaches 3 to 4 times the initial value or when 30 strips have been removed. All collected tapes will be grouped for extraction to maximize efficiency of the subsequent analysis. After tape extraction by methanol, the plant compounds taken up into the skin will be identified and quantified using high performance liquid chromatography coupled with mass spectrometry (HPLC-MS).
Sample size (or equivalent qualitative approach)	5-10 volunteers are required.
Proposed Analysis	The mass of the SC removed on each strip is achieved by weighing an individual tape strip before and after application to the skin. With this information and the TEWL measurement described above, the total thickness of SC can be deduced and, for each the compound identified, the cumulative amount as a function of SC depth expresses its ability to penetrate the SC. Comparison of results using different formulations should ultimately enable the optimal vehicle properties to be determined.
Potential risks to volunteers	1. <u>Topical product application</u>: a. The extracts of *Clerodendrum petasites* S. Moore in creams/lotion (10% w/w) applied to the skin are not expected to cause any

	measurable toxicity. The plant itself has been used in Thai traditional medicine for at least 25 years with no reports of toxicity. b. Excipients in the creams/lotions (e.g., mineral oil and propylene glycol) are suitable and widely used without problems in topical products. Tween 60 (polyethylene glycol sorbitan monostearate) and Span 60 (sorbitan monooctadecanoate) are non-ionic surfactants in the formulations and are hypoallergenic and not expected therefore to cause any measurable toxicity. c. Only crushed leaves and powders of *C. petasites* have been used in traditional medicine. Topical products containing 10% w/w extracts of the plant have not been investigated. The concentrated extracts and excipients in the cream/lotion may result in better typical delivery than traditional preparations. However, data from extensive *in vitro* experiments suggest that the amounts absorbed are far below those reported to cause cytotoxicity. Exposure to the novel formulations may cause redness, and/or dry skin at the site of application and these effects persist for several days. Experiments will be stopped if there is a clear allergic response or a strong inflammatory reaction. Participants are advised of this albeit unlikely possibility in the information sheet. 2. <u>Measurement of TEWL</u>: This technique is completely non-invasive, widely used in skin research, and presents no risk for the participant. 3. <u>Tape-stripping</u>: Removal of the SC disrupts the skin barrier causing some mild irritation, redness and dryness. These effects may persist for several days. Participants will be advised about a basic skin-care treatment after the experiment.
Potential for pain/discomfort	Creams/lotions exposure may induce irritation if a participant is allergic to a particular component in the plant

	or to an excipient. Tape stripping may induce visible redness and dryness of the skin. To minimize this inconvenience, the area of skin involved has been kept to the minimum possible without compromising the limits of quantification of the analytical methods employed.
Benefits to participants	There are no direct benefits to the research participants. The optimized topical formulation may be of general benefit in the future.
How will participants be recruited?	This study will only include healthy subjects, who have no acute or chronic skin complaints, and who will be recruited by word of mouth and by poster advertisement on the campus of University of Bath. They will be drawn from staff and student populations.
Exclusion/inclusion criteria	<u>Inclusion criteria</u> 1. Individuals aged between 18 and 60 years old. 2. Individuals with healthy skin. <u>Exclusion criteria</u> 1. Individuals with a history, no matter how minor, of skin disease. 2. Undergraduate pharmacy students will be excluded because Prof. Richard H Guy and Dr. Michael G Rowan are actively involving in teaching this population. 3. Individuals allergic or particularly sensitive to *C. petasites*, Tween 60 (polyethylene glycol sorbitan monostearate) and Span 60 (sorbitan moonooctadecanoate). 4. Subjects unwilling to agree to the criteria for participation (e.g., refraining from the use of skin-care products during the study).
How will participants consent be taken?	A participant information sheet written in language accessible for a lay population, including a description of risks, will be provided to each potential volunteer. The potential participant will be given as much time as required (a minimum of 24 hours) to decide whether to take part in the study or not. During this period, the researcher will be available to answer any questions and to provide clarifications as required. Written informed consent will be

	obtained from those volunteers that decide to participate.
How will confidentiality be ensured?	No information (e.g., names, initials, dates of birth) that may result in the identification of the participants will be used. Once a volunteer agrees to participate and informed consent is obtained, an identifier code will be assigned that will be used throughout the study. Only these identifiers (subject 1, subject 2, etc.) will be used in publicly-accessible records, graphs, or any other form of communication about the research. Only the PI and principal researcher (Premrutai Thitilertdecha in the first instance) will have access to the actual identities of the participants.

Attach the following (where relevant):

(1) Participant information sheet

(2) Consent Form

(3) Health history questionnaire

(4) Poster/promotional material

(5) Copy of questionnaire/ proposed data collection tool (questionnaire; interview schedule/ observation chart/ data record sheet/ participant record sheet)

Signed by: Principal Investigator or Student Supervisor

_____Date: _____

Signed by: Student or other researchers

_____Date: _____

Considered by SREAP at meeting on:_____

Decision of SREAP:

Action Required:

ANNEX FIVE – Participant Information

It is important to provide clear and easy to understand information about your research to all participants involved in your study. The national research ethics service (formerly COREC) is an organisation within the NHS which has an overseeing function for all NHS Research Ethics Committees. They provide clear and concise information about how to provide information to participants and you should consult the NRES website for up-to-date information on best practice in this area. The website can be found at: WWW.NRES.NPSA.NHS.uk

Information about a research project for potential participants in a project should normally be given in writing on headed letter paper which bears the name of the University and School in which the principal investigator or researcher is based.

This information should include:

- The name of the study
- The name(s) and status(es) of the researchers carrying out the study and how to contact them
- The purpose and value of the study
- Why potential participants are being invited to take part in the research (e.g. because they are residents of particular place, users of a particular facility, members of a particular clinic)
- What the study will involve for participants (description in lay language of all procedures including purposes, duration, location, frequency etc)
- That potential participants can ask questions about the study before they decide whether to participate
- That potential participants can choose whether they participate and, if they agree, they may withdraw from the study without penalty at any time by advising the researchers of this decision.
- That this project has been reviewed by and received ethics clearance through the School for Health Research Ethics Approval Panel (where appropriate)
- Who will have access to personal data provided, how the data will be stored; and what will happen to the data at the end of the project
- What benefits (direct or indirect) may accrue to the participants in the study
- What risks are involved in the study

Tone and Language

You should present this information in a way that is clear and easy to understand for your participants. Try not to use technical language and acronyms unless it is essential to do so. If you do use such terminology, try to give simple and clear explanations of what they mean.

You should be open and friendly in your tone and avoid being overly persuasive as participants should not feel pressurised into participating in your study.

ANNEX SIX – Consent

It is essential that you have a formal record that any participants who are involved in your study have agreed to do so. For each participant you should have a completed 'consent form' which confirms their willingness to participate in your project. The national research ethics service (formerly COREC) is an organisation within the NHS which has an overseeing function for all NHS Research Ethics Committees. They provide clear and concise information about consent and you should consult the NRES website for up-to-date information on best practice in this area. The website can be found at: www.nres.npsa.nhs.uk

Your consent form should be on headed letter paper which bears the name of the University and the name and address of the School for Health.

The consent form should include:

- The name of the study
- The name and status of the researcher(s) collecting the information and how they can be contacted
- The purpose of the study
- Declaration that the participant:
    - Has read the participant information sheet
    - Has had the opportunity to ask questions about the study and has received satisfactory answers to questions, and any additional details requested
    - Understands that s/he may withdraw from the study without penalty at any time by advising the researchers of this decision
    - Understand that this project has been reviewed by, and received ethics clearance through, the School Research Ethics Approval Panel (SREAP) of the University of Bath
    - Understands who will have access to personal data provided, how the data will be stored, and what will happen to the data at the end of the project
    - Agrees to participate in the study

The participant should sign, print and date his/her name. The researcher(s) who secures the consent should sign, print and date her/his name.

ANNEX SEVEN – Frequently asked questions about research ethics approval

The University ethics committee website has a list of links to external resources that you may find helpful when considering the ethical implications of the research work you intend to undertake.

www.bath.ac.uk/internal/ethics/committee/

ANNEX EIGHT – Terms of Reference and Membership of the School Research Ethics Approval Panel

5.1 Purpose

The Purpose of the School Research Ethics Approval Panel is to receive and consider outline proposals for ethical approval of research being undertaken by staff and students of the School for Health.

5.2 The specific ***terms of reference*** of the Research Ethics Approval Panel are to:

- Consider outline research protocols from staff and students of the School to assess the suitability of this work in terms of research ethics (it is envisaged that all proposals that entail work on animal and human subjects will be considered by the Panel, even if the work may not require approval by a local REC – the intention here is to set an ethical standard for all such research work conducted by the School).
- Receive decisions of NRES or other UK or appropriate ethical decision making bodies
- Develop guidance and provide advice to researchers in the School about undertaking ethical research.
- Liaise with the local Research Ethics Committee (and equivalent bodies nationally and internationally) to consider, review and advise on the ethical approval of research being conducted by representatives of the School for Health
- Liaise with the University Ethics Committee in the implementation of the University's research ethics policies
- Share practice with other Ethics Committees which operate within the University (and outside) as appropriate

5.3 Membership of the Panel

The panel will have the following members:

- Member of the REC chair
- Member of Research Committee
- Member of Professional Doctorate Programme Team
- Member of another Department in the University

- A lay member

5.4 Reporting Mechanisms

The School Research Ethics Approval Panel reports directly to the School Executive. Minutes of the Panel will be sent to the School Board of Studies (for student business) and the Research Committee (for staff and research students).

5.5 Frequency of Meetings

The Panel meet on a monthly basis. The frequency of meetings may change based on the volume of proposals to be considered.

Appendix 15.2 Participant information sheet.

Department of Pharmacy & Pharmacology

Claverton Down

Bath, BA2 7AY

Prof. Richard H Guy, PhD

Tel.+44.1225.384901;

Fax +44.1225.386114;

Email: R.H.Guy@bath.ac.uk

Participant information sheet

In vivo assessment of a topical application of

Clerodendrum petasites S. Moore.

- You are being invited to take part in a research study.
- Before you decide on your participation, it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully and discuss with others if you wish.
- Please contact us if there is anything that is not clear or if you would like more information. Our contact details are at the end of this document.
- Take as much time as you want to decide whether or not you wish to take part in this study.

Thank you for reading this information sheet.

Purpose of the study:

Clerodendrum petasites S. Moore (English name: One root plant, Thai name: Thao Yai Mom) is a Thai medicinal plant and has been traditionally used for various indications for at least 25 years. This project primarily focuses on topical use for the treatment of skin problems, such as rash. Scientific knowledge about the plant is sketchy and the products on the market are very variable in quality, and efficacy. However, there have been no reports of toxicity.

In our preliminary investigations on the plant extract, we found that *C. petasites* contains several potentially active substances, mainly polyphenolic compounds which are known as strong antioxidants. These are generally beneficial to health and have been

widely used in the pharmaceutical and cosmetic fields; for example, to prevent of ultraviolet (UV) – induced skin aging, to treat hyperpigmentation and to reduce inflammation. Thus, we expect that the natural molecules discovered in our preliminary studies will provide scientific support for the traditional topical uses of the plant. To confirm this idea, the ability of the naturally-occurring chemicals to penetrate the skin, and particularly the stratum corneum (SC, the outermost, barrier layer of the skin), needs to be demonstrated.

The main objective of this project is to study "which one" and "how much" of the natural substances in creams/lotions containing *C. petasites* extracts (10%w/w) are able to cross the skin and "how far". The collected data will also be useful for further topical product development to improve the reliability of the medicinal formulation.

Creams/lotions are freshly formulated just for this study. All excipients in the formulations are commonly used in topical products and are hypoallergenic.

Volunteer requirements:

For your own safety, and the safety of the researchers, to participate in this study, you should:

1. Be healthy and have not undertaken any skin treatment (e.g., visited a tanning salon), or participated in another experiment, within the last 4 weeks.
2. Not suffer from any skin disease.

Your participation in the research:

It is up to you to decide whether or not to take part in this study. If you decide to take part:

1. You will be given a copy of this information sheet to keep for your records.
2. You will be asked to sign a consent form.
3. If you change your mind, you are still free to withdraw at any time without giving a reason.

What is going to happen to you during the study:

- Your participation involves application of 10% w/w *C. petasites* extract in creams/lotions at four places on the inner side of your forearms for 6 hours per day on each of 2 days. Altogether, each day's experiment will last no more than 8-10 hours.
- Creams/lotions used in the study will be applied to your forearm in the morning at the University of Bath on weekdays except bank holidays.
- After the 6 hours treatment, samples of the SC will be taken from the application sites by a procedure known as tape stripping (see later for a detailed description).

- The experiments will take place in the Department of Pharmacy & Pharmacology at the University of Bath.
- During this experiment, you will not be allowed to eat while the tape stripping procedures are performed. However, you can have a lunch break (as shown in Table 1) and, after the study, biscuits and liquid refreshment will be provided.
- You should not apply any cream, cosmetics or washing products to either of your forearms during your participation in the study.

Treatment site preparation before drug application:

1. A whole-day experiment will be performed on one forearm per day for 2 days as shown in Table 1.

	Morning (Apply drug)				LUNCH	Afternoon (Remove drug and tape strip)			
	Site 1	**Site 2**	**Site 3**	**Site 4**		**Site 1**	**Site 2**	**Site 3**	**Site 4**
Day1, Forearm 1	9.00	9.45	10.30	11.15		15.00	15.45	16.30	17.15
Day2, Forearm 2	9.00	9.45	10.30	11.15		15.00	15.45	16.30	17.15

Table 1. Experiment design over 2 days.

2. We will clean the forearm with an alcohol swab wipe (like that used before an injection) and let the skin dry completely.
3. Four treatment sites (2 cm x 3 cm) will be dermacated on the arm with a rectangular self-stick adhesive foam frame (1.57mm thick, 3M, USA), as illustrated in Fig. 1.

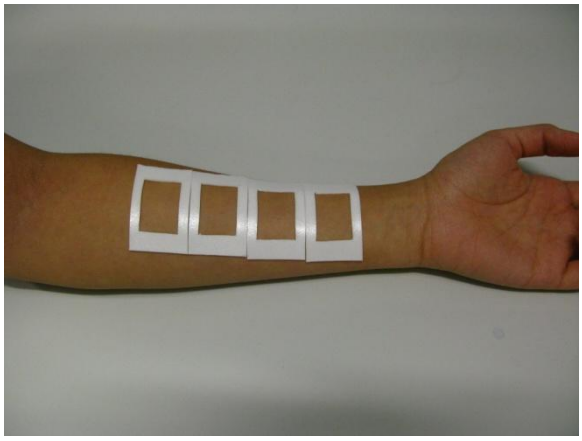


Fig. 1. Treatment sites on each forearm

4. One tape strip (2.5cm x 2.5cm, Permacel J-LAR®) will be placed on your skin and then removed in one swift motion (Fig. 2). This step does not cause any discomfort to the skin because only the very outermost layer is removed.

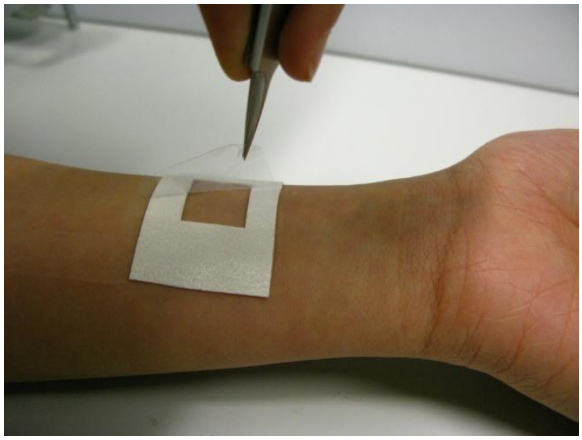


Fig. 2. Tape stripping

5. Between tape-strips, a small probe (Fig. 3) will be rested on your skin to measure how quickly water is escaping through the treated areas. These measurements, which cause no sensation at all, allow us to determine for how long tape-stripping should continue.

Fig. 3. Measurement of water loss through the skin

Drug application:

1. Cream/lotion containing the plant extract (10%w/w) will be applied at sites 1, 2 and 3.
2. The cream/lotion without the plant extract will be applied at site 4.
3. Formulation will be applied successively to the 4 sites at 45-minute intervals (Table 1), then covered with a plastic film, and held to the skin with a self adhesive dressing (Mefix®), as shown in Fig. 4.

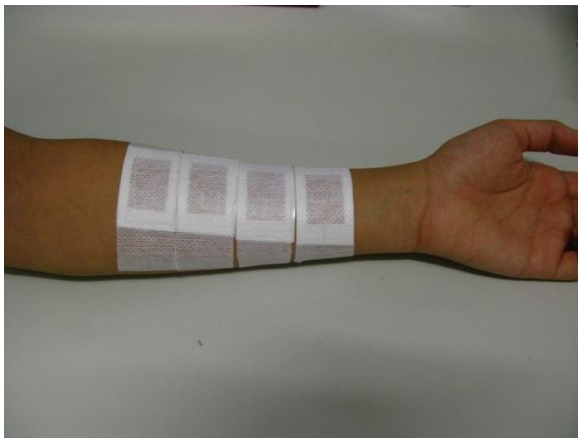


Fig. 4. Drug application

4. Creams/lotions will contact your skin for 6 hours at each site.
5. Subsequently, we will remove the dressing, the film, and the frame.

6. Excess cream/lotion left on your skin will be gently wiped away with tissue (Fort James Ltd., UK) and alcohol wipes (Steret®) to clean the skin before the tape stripping process.

Tape stripping procedures:

1. A new, smaller foam frame will be placed at the end treated skin site as the first one (Fig. 5) to deliniate the area for tape stripping.

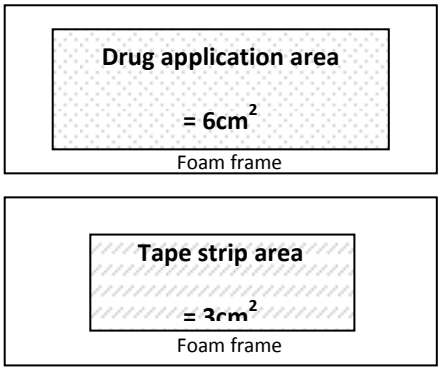


Fig. 5. Illustration of drug application and tape strip areas.

2. Water loss through the skin will be measured as before (Fig. 3).

3. One tape strip will be placed on the site and removed with a swift motion (Fig. 5). Water loss is measured again.

Fig. 6. Tape stripping

4. Step 3 will be repeated no more than 30 times and will cause no more than slight discomfort. There is no possibility, for example, of any blood loss.

- During your participation in the experiments, you will remain seated. In the laboratory you will not be allowed to eat or smoke, nor drink anything except water. You are, of course, permitted have lunch or to take a comfort break outside the laboratory after the drug application process and before tape stripping begins.
- Should you, at any time, experience discomfort or if you feel unwell, the experiment will be stopped.
- At the end of the procedures, we will ask you to complete a very short questionnaire about the level of sensation/discomfort that you experienced during the experiment. We will also ask you about your history of any skin disease as this may help us to interpret our results.

Possible adverse effects:

1. The topical products containing 10% w/w *C. petasites* extract may cause some irritation at the site of application. The symptoms may persist for several days.
2. The removal of the outer layer of the skin by tape stripping can cause redness and dryness. These effects may persist for several days. Tape stripping is a benign procedure but may provoke mild discomfort once several strips have been removed.

Possible benefits of taking part:

There is no direct benefit for you as a result of your participation. This is a basic research study designed to help us develop a topical medicinal product to treat skin disease or for cosmeceutical purposes (e.g. anti-oxidation).

If something goes wrong:

The University of Bath has insurance to cover research on healthy volunteers.

Recognition of your time:

To thank you for your participation and time, we will pay you £75 at the end of the experiment. You will receive this amount even if the experiment is stopped before completion; for example, if you experience unacceptable discomfort, or if you feel unwell, during any of the procedures performed.

Confidentiality:

All personal information collected during the course of this research will be kept strictly confidential. When the results are made public, they will not include any names, initials or any type of information which could result in your identification. The researcher may ask for permission to take some photographs during the experiment. If you agree, the pictures will **only** include your treated forearm.

Results of the study:

The results of this study will be published in scientific journals and/or presented during conferences and/or internal reports. You will be provided with a copy of any published articles upon request.

Organization and funding of the research:

This research is funded by the Faculty of Medicine Siriraj Hospital, Mahidol University, Thailand.

The protocol has been reviewed by the School for Health Research Ethics Approval Panel.

Contact for further information:

We will be more than happy to answer any questions you have about this research and your participation.

Please contact:

Ms. Premrutai Thitilertdecha: pt245@bath.ac.uk	Tel. 01225 383900
Prof. Richard H Guy: r.h.guy@bath.ac.uk	Tel. 01225 384901
Dr. Michael G Rowan: prsmgr@bath.ac.uk	Tel. 01225 386789

You will be given a copy of this information sheet and a signed consent form to keep.

Thank you for your interest in this study

Appendix 15.3 Consent form.

UNIVERSITY OF BATH

Department of Pharmacy & Pharmacology

Claverton Down

Bath, BA2 7AY

Prof. Richard H Guy, PhD

Tel.+44.1225.384901;

Fax +44.1225.386114;

Email: R.H.Guy@bath.ac.uk

Centre: University of Bath, Department of Pharmacy & Pharmacology,

Skin & Nail Research Group

Participant Identification Number for this study:

CONSENT FORM

In vivo assessment of a topical application of

Clerodendrum petasites S. Moore.

Researcher: Ms. Premrutai Thitilertdecha

Principal Investigators: Prof. Richard H Guy and Dr. Michael G Rowan

1. I confirm that I have read and that I understand the Participant Information sheet dated November 3, 2011 for this study and that I have had the opportunity to ask questions.	
2. I understand that my participation is voluntary and that I am free to withdraw at any time, without giving any reason, without my medical care or legal rights being affected.	
3. I understand that this project has been reviewed by, and received ethics clearance through, the School Research Ethics Approval Panel (SREAP) of the University of Bath	
4. I understand that all personal information collected during the course of this research will be kept strictly confidential. When the results are made public, they will not include any names, initials or any type of information which could result in your identification. Only the principal investigators	

(Richard H Guy and Michael G Rowan) and the direct researcher (Premrutai Thitilertdecha) will have access to the real names of participants.	
5. I allow the researchers to take photographs of my forearms during the experiment.	
6. I have not participated in any other skin study in the last 2 months and, if I did, I have informed the researcher of the nature of the study.	
7. I agree to take part in the above study.	

_____ _____ _____

Name of Participant Date Signature

_____ _____ _____

Researcher Date Signature

Appendix 15.4 Questionnaire.

UNIVERSITY OF BATH

Department of Pharmacy & Pharmacology

Claverton Down

Bath, BA2 7AY

Prof. Richard H Guy, PhD

Tel.+44.1225.384901;

Fax +44.1225.386114;

Email: R.H.Guy@bath.ac.uk

Centre: University of Bath, Department of Pharmacy & Pharmacology,

Skin & Nail Research Group

Participant Identification Number for this study:

Questionnaire

Date:.....

Participant Identification Number:.....

Forearm used: .....

Gender of the participant:.....

Age of the participant:.....

Formulation Number:.....

Part I: Skin disease history.

1. Have you had any form of skin disease? Yes / No

(If yes, please give details below)

.....

2. Have you participated in any other experiments irritating skin in the last 2 months?

Yes / No (If yes, please give details below)

.....

3. Would you describe your skin as:

- ☐ Never feels dry
- ☐ Occasionally feels dry
- ☐ Only feels dry in certain months of the year
- ☐ Constantly feels dry

4. Do you suffer from any skin allergies, asthma or allergic rhinitis? Yes / No

(If yes, please give the details below)

.....

Part II: Sensation and discomfort during experiment.

Express your degree of sensation and discomfort using the scale from 0 to 5. Please indicate 0 if you did not feel any discomfort, 5 if you felt very strong discomfort.

1. Discomfort during drug application at the skin sites:

Site 1	0	1	2	3	4	5
Site 2	0	1	2	3	4	5
Site 3	0	1	2	3	4	5
Site 4	0	1	2	3	4	5

2. Discomfort during tape stripping at the skin sites:

Site 1	0	1	2	3	4	5
Site 2	0	1	2	3	4	5
Site 3	0	1	2	3	4	5
Site 4	0	1	2	3	4	5

3. In your opinion, was your skin irritated during any part of experiment? Yes / No

(If yes, please give details about your sensations and discomfort below)

.....

Please let us know if you have any comments about the experiment:

.....

Thank you for your participation.